



(12) **EUROPEAN PATENT APPLICATION**

(43) Date of publication:  
07.05.2003 Bulletin 2003/19

(51) Int Cl.7: **C12N 1/20**, A01N 63/00,  
A23K 3/00, A23L 3/3571,  
C12R 1/245, C12R 1/01

(21) Application number: **01125464.6**

(22) Date of filing: **06.11.2001**

(84) Designated Contracting States:  
**AT BE CH CY DE DK ES FI FR GB GR IE IT LI LU**  
**MC NL PT SE TR**  
Designated Extension States:  
**AL LT LV MK RO SI**

(72) Inventors:  
• **Miescher Schwenninger, Susanne**  
**8092 Zürich (CH)**  
• **Melle, Leo**  
**8092 Zürich (CH)**

(71) Applicant: **Eidgenössische Technische**  
**Hochschule Zürich**  
**8092 Zürich (CH)**

(74) Representative: **Blum, Rudolf Emil Ernst et al**  
**c/o E. Blum & Co**  
**Patentanwälte**  
**Vorderberg 11**  
**8044 Zürich (CH)**

(54) **Mixtures of *Propionibacterium jensenii* and *Lactobacillus* sp. with antimicrobial activities for use as a natural preservation system**

(57) The invention concerns a mixture of bacteria. Said mixture is a non starter culture which is free from metabolites and comprises at least one first bacterium selected from the species *Propionibacterium jensenii* and at least one second bacterium selected from the genus *Lactobacillus*.

Furthermore, food, feeding stuff and medicaments comprising such a mixture, a method for manufacturing and storing such goods and the use of the mixture to inhibit fungi and bacteria are provided.

## Description

[0001] The invention concerns bacteria suitable for the preservation of goods, in particular a mixture of bacteria according to claim 1. Furthermore, the invention concerns food, feeding stuff or a medicament according to claim 12 comprising such a mixture, a method for manufacturing such food, feeding stuff or medicament according to claim 14, a method for storing such food, feeding stuff or medicament according to claim 17 and the use of such a mixture according to claim 18.

[0002] Outgrowth of undesired microorganisms in food products can be counteracted by different ways. Beside physical and chemical treatments, biological treatments such as fermentations by naturally resident and/or artificial ("starter") microorganisms are widely used. They can change the food conditions in which growth of undesirable organisms is less favourable or completely inhibited as is the case in wine, beer, cheese or yoghurt (Holzapfel *et al.* 1995; Vogel, 1996). Another system of biologically induced food preservation is the supplementation of "protective cultures" acting as "biopreservatives". Such cultures are thought

- a) either to grow and competitively suppress undesired organisms
- b) or to grow and produce antimicrobial and antifungal agents (e.g. bacteriocins, organic acids, diacetyl, unknown metabolites)
- c) or to interact by unknown mechanisms with food spoiling microorganisms.

Since protective cultures generally do not participate in specific food modification processes as starter cultures, their application can also be extended to nonfood materials.

[0003] Antimicrobial activities of propionibacteria and lactic acid bacteria make them appropriate for industrial application as biopreservatives. Bio Profit (Valio Ltd., Helsinki, Finland; Wiesby GmbH & Co., Niebüll, Germany), is a commercially available co-culture of *Lactobacillus rhamnosus* LC705 (DSM 7061) (former *Lactobacillus casei* subsp. *rhamnosus*) and *Propionibacterium freudenreichii* subsp. *shermanii* JS (DSM 7067), that is suggested for a controlling of yeasts and moulds (Soumalainen and Mäyrä-Mäkinen, 1999). Both organisms had been cultured together and were supposed to be used as a cell containing fermentation broth inhibiting the growth of moulds and yeasts in food. Strain *Lactobacillus rhamnosus* LC705 was protected in 1993 by a European patent (EP 0 576 780) to be used as a single strain or in combination with a bacterium of the genus *Propionibacterium* or another strain of the bacterial species *Lactobacillus casei*. A second, German patent (DE 199 17 715) describes protective cultures consisting of lactic acid bacteria and inhibiting the growth of toxigenic bacteria at temperatures over 7-8°C. The cultures are suggested for a preservation of food and animal food (feeding stuff) showing only a short shelf-life below 7-8°C. As soon as the storage temperature increases, the cultures promise to inhibit the growth of spoilage bacteria. In contrast, Microgard™ (Wesman Foods, Inc., Beaverton, Oregon, USA) is a commercially available milk product fermented by a *P. freudenreichii* ssp. *shermanii* strain, followed by a pasteurisation process. The product promises effectiveness in inhibiting selected food spoilage organisms as well as pathogenic microorganisms known to cause foodborne illnesses (Dae-schel, 1989; Al-Zoreky *et al.*, 1991). Microgard™ is approved by the US Food and Drug Administration.

[0004] Based on the prior art, it is the object of the invention to provide an improved bacteria based inhibitor for the growth of fungi (yeasts, moulds) and/or bacteria as well as mixtures thereof.

[0005] This object is solved by the independent claims.

[0006] It was unexpectedly found that bacteria selected from the species *Propionobacterium jensenii* and/or the genus *Lactobacillus* that are non-starter cultures and free from metabolites are suitable inhibitors of undesired microorganisms. In particular a mixture of bacteria (also referred to as inhibiting mixture or protective culture) - which is a non starter culture - and which is free from metabolites and that comprises at least one first bacterium selected from the species *Propionibacterium jensenii* and at least one second bacterium selected from the genus *Lactobacillus*, is able to inhibit at least one microorganism selected from fungi and/or bacteria as well as mixtures of fungi and bacteria which for example are responsible for the deterioration of food and feeding stuff.

[0007] This mixture of bacteria has the great advantage that - although metabolites that hitherto were thought to be responsible for protective activity are absent - the protection is as good or even better than with hitherto known cultures and undesired side effects due to metabolites (e.g. the metabolites can be toxic or also negatively influence the taste, colour of food etc.) are markedly reduced or even fully eliminated.

[0008] The mixture of bacteria is incorporated into and/or applied onto the surface of the good to be protected.

[0009] The term "metabolites" comprises all products which origin from the metabolism of the bacteria comprised by the mixture.

[0010] In this application, bacteria and fungi which are able to deteriorate food, feeding stuff, medicaments are also called "unwanted microorganisms".

[0011] The term "a mixture of bacteria free from metabolites" means that only the mixture of bacteria (e.g. separated from metabolites at least by centrifugation, preferably by centrifugation and at least one washing step), optionally

together with a carrier, is incorporated into and/or applied onto the surface of the good (food, feeding stuff, medicament) to be protected from deterioration.

[0012] Furthermore, in connection with this patent application, the term "to be inhibiting, to inhibit etc." means for example that the growth or also the number or the concentration of unwanted microorganisms, for example in food and/or onto the surface of food comprising the mixture, is lower than in food and/or onto the surface of food which does not comprise such a mixture.

[0013] The term "a mixture which is a non starter culture" means a mixture that comprises bacteria which are not or not essentially adapted to the good - in particular food, feeding stuff, medicaments - to be protected by the mixture such that they do not or only minimally grow. Minimal growth means a growth of up to at most about tenfold.

[0014] A preferred mixture is a mixture of bacteria as described above that is obtainable by the process which comprises the following steps:

[0015] Firstly, an inhibiting mixture which comprises at least one first and at least one second bacterium and which mixture is free from metabolites is incorporated into a medium and/or applied onto the surface of a medium contained in a first container such that a minimum concentration as defined further below of each of said at least one first and at least one second bacteria in this medium and/or on the surface of this medium results.

[0016] The medium contained in the first and second container (the second container is described below) in or on which the inhibition of the contaminants is tested preferably is food or feeding stuff or a medicament, or much preferably solid agar medium.

[0017] The at least one first bacterium is selected from the species *Propionibacterium jensenii* and the at least one second bacterium is selected from the genus *Lactobacillus*.

[0018] As a blank, a second container is prepared comprising the same medium as said first container but no mixture of bacteria.

[0019] Then the same number of contaminants are incorporated into the medium and/or applied onto the surface of the medium contained in said first and second containers.

[0020] The term "contaminants" is equal to the term "unwanted microorganisms" and comprises fungi and/or bacteria as well as mixtures of fungi and bacteria.

[0021] The inhibiting effect of the mixture against the contaminants is determined by storing the first and second container at a suitable temperature during a suitable storage time.

[0022] In general, said suitable temperature at which this method is performed depends on the temperature at which a specific good, in particular food, feeding stuff or medicament normally is stored and/or manufactured.

[0023] The temperature at which the first and second container are usually stored is 5-26°C, preferably said temperature is at least selected from the group of approx. 6°C and/or approx. 12°C and/or approx. 25°C.

[0024] The term "approx." means  $6 \pm 1^\circ\text{C}$ ,  $12 \pm 1^\circ\text{C}$ ,  $25 \pm 1^\circ\text{C}$ .

[0025] The storage time at said temperature depends on the time during which the good (food, feeding stuff or a medicament) normally is stored.

[0026] The storage time usually is 7-28 days, preferably said storage time is selected at least from the group of 7 days and/or 14 days and/or 21 days and/or 28 days.

[0027] During said storage time at a specific temperature, the number or the concentration of contaminants incorporated into the medium and/or applied onto the surface of the medium contained in the first and second container is compared.

[0028] Based on this comparison (number/concentration of contaminants in said first container at a specific time versus number/concentration of contaminants in said second container at that specific time), a first and a second graph can be drawn in a coordinate system where e.g. the x-axis represents the storage time and the y-axis represents the number and/or concentration of contaminants. The first graph shows the number or concentration of the contaminants incorporated into the medium and/or applied onto the surface of the medium in the first container and the second graph shows the same measure in the medium and/or on the surface of the medium contained in the second container.

[0029] If only single strains or mixtures of strains of the same species or genus shall be investigated, the above mentioned method can be used accordingly.

[0030] The mixture is considered to be inhibiting during the stages of the storage time, if said second graph is above said first graph.

[0031] Preferably, the mixture is considered to be inhibiting if the number of contaminants in the medium and/or on the surface of the medium contained in the second container is at least about log 1 higher, preferably at least about log 2, much preferably at least about log 3, more preferably at least about log 4, even more preferably at least about log 5, most preferably at least about log 6 than the number of contaminants in the medium and/or on the surface of the medium contained in said first container during at least 3 days, preferably at least 7 days, much preferably at least 14 days, more preferably at least 21 days, most preferably 25 days.

[0032] According to a preferred embodiment of the invention, the at least one first bacterium mentioned above is *Propionibacterium jensenii* SM11.

[0033] According to another preferred embodiment of the invention, the at least one second bacterium mentioned above is selected from the group consisting of *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus plantarum*, and mixtures thereof.

[0034] According to a further, more preferred embodiment of the invention, the at least one second bacterium is selected from one or more strains of *Lactobacillus paracasei* subsp. *paracasei*.

[0035] According to an even more preferred embodiment of the invention, the at least one second bacterium is *Lactobacillus paracasei* subsp. *paracasei* SM20 and/or *Lactobacillus paracasei* subsp. *paracasei* SM29 and/or *Lactobacillus paracasei* subsp. *paracasei* SM63, and mixtures thereof.

[0036] The ratio between the at least one first bacterium and the at least one second bacterium of the mixture - e.g. the ratio of the concentration or number of the at least one first bacterium and the concentration or number of the at least one second bacterium - preferably amounts from 1:100 - 100:1, preferably 1:10 - 10:1.

[0037] The mixtures described above provide the advantage that microorganisms selected from the group consisting of fungi, bacteria and mixtures of fungi and bacteria which are or can be pathogenic for humans and/or animals and/or which have or can have a spoilage effect - for example on food, feeding stuff, medicaments - can be inhibited.

[0038] For example in case of food - means for preservation such as chemical processes (e.g. the application of food preservatives, salting) and/or physical processes (e.g. heat, UV-rays, Gamma-rays, x-rays), packaging using protective gases (e.g. N<sub>2</sub>, CO<sub>2</sub>) or vacuum packaging can be reduced or totally omitted without reduction of the storage time / shelf life of the food.

[0039] As a consequence thereof, especially low processed food or also low processed ingredients which for example are added to high processed food can be stored during a longer period without biological deterioration or also can be better exported to other countries.

[0040] If desired, the mixture can also be used in connection with the preservation processes mentioned above.

[0041] Using such preservation processes in combination with the mixture, it is a prerequisite that the mixture still can perform its protective purpose.

[0042] This means for example that after the application of said preservation process, the good to be protected still has to comprise at least the following minimum concentrations of viable cells (cfu = colony forming unit).

[0043] The minimum concentration of the at least one first or the at least one second bacterium in the food in view of protection amounts at least to about  $1 \times 10^7$  cfu/ml, preferably at least to about  $1 \times 10^8$  cfu/ml, even more preferably at least to about  $1 \times 10^9$  cfu/ml or at least to about  $1 \times 10^7$  cfu/g, preferably at least to about  $1 \times 10^8$  cfu/g, even more preferably at least to about  $1 \times 10^9$  cfu/g and/or on the surface of the food at least to about  $1 \times 10^6$  cfu/cm<sup>2</sup>, preferably at least to about  $1 \times 10^7$  cfu/cm<sup>2</sup>, much preferably at least to about  $1 \times 10^8$  cfu/cm<sup>2</sup>, even more preferably at least to about  $1 \times 10^9$  cfu/cm<sup>2</sup>.

[0044] If higher activity against contaminants is desired, concentrations of at least about  $1 \times 10^{10}$  cfu/ml or at least about  $1 \times 10^{10}$  cfu/g and/or at least about  $1 \times 10^{10}$  cfu/cm<sup>2</sup> are possible.

[0045] The minimum concentrations mentioned above go out from the experimental observation that the higher the concentration or number of the protective culture is, the more the fungi and/or bacteria as well as mixtures thereof are inhibited.

[0046] While some protection is already observed if the first and second bacteria are present in amounts of  $1 \times 10^7$  cfu/ml or cfu/g, the protection is much better if the at least one first bacterium and the at least one second bacterium combined with each other to the mixture of bacteria amount at least to about  $5 \times 10^7$  cfu/ml or at least to about  $5 \times 10^7$  cfu/g and/or at least to about  $5 \times 10^6$  cfu/cm<sup>2</sup> for the at least one first bacterium and at least to about  $1 \times 10^8$  cfu/ml or at least to about  $1 \times 10^8$  cfu/g and/or at least to about  $1 \times 10^7$  cfu/cm<sup>2</sup> for the at least one second bacterium. Very satisfying results were achieved with at least about  $1 \times 10^8$  cfu/ml or at least about  $1 \times 10^8$  cfu/g and/or at least about  $1 \times 10^7$  cm<sup>2</sup> for the at least one first bacterium and at least about  $1 \times 10^8$  cfu/ml or at least about  $1 \times 10^8$  cfu/g and/or at least about  $1 \times 10^7$  cm<sup>2</sup> for the at least one second bacterium. Presently preferred are at least about  $5 \times 10^8$  cfu/ml or at least about  $5 \times 10^8$  cfu/g and/or at least about  $5 \times 10^7$  cfu/cm<sup>2</sup> for the at least one first bacterium and at least to about  $5 \times 10^8$  cfu/ml or at least about  $5 \times 10^8$  cfu/g and/or at least about  $5 \times 10^7$  cfu/cm<sup>2</sup> for the at least one second bacterium.

[0047] The minimum concentrations and preferred concentrations mentioned above apply also to medicaments and feeding stuff.

[0048] The determination of the surface concentration can be performed by two methods:

(i) by measuring the applied amount, e.g. in spraying application, or preferably

(ii) by measuring the concentration of a specific volume with well defined application surface and comprising the whole diffusion zone of the mixture of bacteria.

[0049] The term "at least one first bacterium of  $1 \times 10^7$  cfu/ml" means that a first bacterium and optionally one or more other first bacteria which are different from each other and which all belong to the genus *Propionibacterium*

*jensenii* are simultaneously present, whereby all said first bacteria together form a concentration of  $1 \times 10^7$  cfu/ml. This definition also applies to equivalent passages in the application.

[0050] The minimum concentrations defined above have to be given in any stage after the completion of the manufacturing process of the food, feeding stuff or medicament.

[0051] In any case, the concentration of the mixture must be higher than or at least equal to the minimum concentration before the beginning of the storage.

[0052] Preferably, food, feeding stuff or medicaments comprise such a concentration of the bacteria of the mixture that the concentration of the bacteria and/or fungi or mixture of bacteria and fungi is kept below the requirements mentioned in the food regulation of the respective country where this invention is used.

[0053] Preferably, but not necessarily, these minimum concentrations also apply during the performance of the manufacturing method.

[0054] If the concentration of the bacteria of the mixture in the good and/or onto the surface of the good to be protected falls below the minimum concentration during the performance of the manufacturing method because of for example the application of preservation processes which reduce the viable cells (heat, UV-rays etc.) or as a consequence of concentration changes during the manufacturing (e.g. by addition of further ingredients etc.) cells can be added during the performance of the manufacturing. In case of a manufacturing method comprising a long lasting step, it is important that the minimum concentration is already present during said step such as for example during the ripening of sausages.

[0055] If a reduction of the concentration of viable cells takes place during the storage such that the required minimum concentration is not given any more, additional viable cells have to be added if possible.

[0056] In general, the concentration of the mixture of bacteria in food, feeding stuff and medicaments can be higher than the minimum concentrations mentioned above. But the concentrations have to be selected such that the food, feeding stuff or the medicament is not affected by undesired deteriorations. This means for example that the mixture does not influence the sensory or other quality properties of the food (e.g. discoloration etc.).

[0057] Besides the mixtures described above, the invention also provides a method for manufacturing e.g. feeding stuff, medicaments, and preferably food, comprising such mixtures.

[0058] This manufacturing method allows that unwanted microorganisms are not able to grow.

[0059] In the following, this method is described by means of food, but this does not exclude that the following teaching can also be applied to the manufacturing of other goods, e.g. feeding stuff and medicaments.

[0060] The method comprises the following steps: Firstly, the mixture is added during the manufacturing of the food in an amount such that the concentration of the at least one first bacterium and the at least one second bacterium in the food each amounts at least to the minimum concentration cfu/ml or cfu/g of the food and/or at least to the minimum concentration cfu/cm<sup>2</sup> of the surface of the food.

[0061] In general, the mixture has to be added at a stage of the manufacturing method or prior to the storage such that it can be evenly distributed in the good and/or onto the surface of the good to be protected.

[0062] The mixture preferably is applied at a stage prior to a significant contamination with unwanted microorganisms.

[0063] In a second step of the manufacturing, e.g. after the addition of the mixture, one or more parameters of the manufacturing method have to be controlled such that the concentration of the mixture decreases or, preferably remains constant. While decrease in concentration can be "healed" as described above, significant growth must be avoided.

[0064] Such parameters are for example the temperature, pressure or also the ingredients of the food etc.

[0065] By the term "constant" it is intended - once the mixture is added to the good, e.g. food or feeding stuff or medicament - that the concentration of the mixture remains constant. Such constant mixture preferably is added in an amount such that at least the minimum concentration as defined in this application results. By the term "constant", is also intended an increase of number or concentration of the bacteria of the mixture by means of growth of the bacteria up to at most tenfold.

[0066] In case the manufacturing method comprises one or more fermentation steps, the mixture - if added prior to the fermentation - must not have a negative influence on the microorganisms (e.g. bacteria, moulds, yeasts or mixtures thereof) responsible for said one or more fermentations (e.g. reduction of the fermentation activity).

[0067] If the mixture has such a negative influence on these microorganisms, the mixture is added after said one or more critical fermentation steps or after the completion of the manufacturing method.

[0068] Also in manufacturing methods comprising steps that might be affected by the mixture of bacteria, said bacteria are added after such one or more steps.

[0069] Furthermore, the invention provides a method for storing goods, in particular feeding stuff, medicaments and preferably food wherein the growth of unwanted microorganisms during storage is inhibited.

[0070] This method for storage can be used for the storage of e.g. food, feeding stuff or medicaments which are manufactured according to the manufacturing method of the invention or by known manufacturing methods.

[0071] Only during the manufacturing method according to the invention, the parameter or parameters of the manufacturing method are controlled such that the concentration of the mixture remains constant.

[0072] Using other e.g. common manufacturing methods, there is no or only partial such control. Therefore, the

mixture should be added in such manufacturing method at a stage after which the concentration of the mixture remains constant.

[0073] The method for storing comprises the step of controlling the storage parameter or storage parameters during the storage such that the concentration of the at least one first and at least one second bacterium remains constant.

[0074] Possible storage parameters are for example the temperature, storage atmosphere around the good, storage atmosphere within the packaging of the good etc.

[0075] The term "storage" means the time after completion of the manufacturing of the good, e.g. food, feeding stuff or medicament and therefore said term for example can also comprise transportation.

[0076] The mixture of bacteria according to the invention preferably is contained in and/or applied onto the surface of food, feeding stuff or medicaments.

[0077] Such food preferably are dairy products, more preferably sour milk products.

[0078] Furthermore, meat and meat products are also preferred.

[0079] Dairy products are for example raw milk, heat-treated or microfiltrated milk, butter, curdled milk, sour cream, skim milk, cream, yoghurt, buttermilk, curd, cheese, kefir, kumiss, puddings, sour milk, acidophilus sourmilk, langfil (ropy milk), bifidus sourmilk, junket, sour cream butter.

[0080] Meat is for example portioned fresh beef or poultry meat or minced meat.

[0081] Meat products preferably are ready to eat sausages, pet food, nitrite and nitrate cured meat (pickled raw meat), cooked salt meat, raw sausage etc.

[0082] Products like for example delicatessen salads made of meat, fish, molluscs, crustaceans, vegetable, pasta and mixtures thereof, but also fish, fish products, molluscs, crustaceans, fruits, fruit products, nuts, nut products, and vegetables such as potatoes, vegetable products, corn, corn products can be protected by the mixture.

[0083] Possible combinations of the food mentioned above comprising the mixture according to the invention are also felt to fall under the present invention.

[0084] Feeding stuff preferably are products derived from nuts, corn, gras.

[0085] Medicaments which can be protected by the mixture are natural and organic remedies.

[0086] According to one embodiment of the invention, food, feeding stuff or medicaments are treated with a powder comprising the mixture which powder optionally comprises suitable carrier substances. Suitable carrier substances are for example saccharides, preferably monosaccharides, disaccharides or their derivatives.

[0087] According to a further preferred embodiment of the invention, food, feeding stuff or medicaments are treated with a liquid medium comprising the mixture. Such a liquid medium must be designed such that it guarantees the survival of the mixture. It can be for example an aqueous medium such as a liquid milk product or a physiological sodium chloride solution.

[0088] According to a preferred embodiment of the invention, the mixture is incorporated into the food, feeding stuff or medicament by mixing, spraying etc., whereby by spraying or another suitable type of application such as dipping, the mixture is applied onto the surface of food, feeding stuff or medicament.

[0089] In case of dairy products, the mixture preferably is comprised in a liquid, whereby the liquid is added to the good to be protected. The mixture can also be added as a deep freeze-dried or freeze-dried concentrate.

[0090] The mixture according to the invention that is preferably incorporated into and/or applied on the surface of food, feeding stuff or medicaments, more preferably into and/or on those specified above, allows in particular the inhibition of bacteria such as *Listeria innocua*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Serratia liquefaciens*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Enterobacter cloacae*, *Enterococcus faecalis*, *Bacillus subtilis*, *Bacillus cereus*, *Bacillus anthracis* and mixtures of these bacteria, lower eukaryotes such as algae and mixtures of lower eukaryotes, fungi such as *Candida magnoliae*, *Candida parapsilosis*, *Candida silvicola*, *Candida valida*, *Candida pulcherrima*, *Candida reukaufii*, *Candida krusei*, *Candida sp.*, *Sporobolomyces salmonicolor*, *Zygosaccharomyces bailii*, *Penicillium sp.*, mixtures of these fungi as well as mixtures of the bacteria and fungi mentioned above as well as mixtures of the bacteria and fungi and lower eukaryotes mentioned above.

[0091] The invention is not considered to be limited to the bacteria, fungi, food, feeding stuff and medicaments explicitly mentioned in this application.

[0092] Further embodiments, advantages and applications of the invention result from the dependent claims and the following examples making reference to the figures:

Fig. 1 shows a composition of 82 isolates on MRS agar originating from raw milk, cheese, yoghurt, black olives, salami as well as mais and gras silage showing antifungal activities in an agar spot assay and being identified with API 50 CHL. <sup>a</sup> identified as *Lactobacillus paracasei* subsp. *paracasei* analysing the 16S rRNA gene.

Fig. 2 shows a 2 % agarose gel of the amplification of a 290-bp product obtained using the *casei*-group-specific oligonucleotides in conjunction with primer Y2 (Table 2). (A) *Lactobacillus paracasei* subsp. *paracasei* SM20; (B) *Lactobacillus paracasei* subsp. *paracasei* SM29; (C) *Lactobacillus paracasei* subsp. *paracasei* SM63; (D) *Lactobacillus casei* DSM 20011<sup>T</sup>; (E) *Lactobacillus paracasei* subsp. *paracasei* DSM 5622<sup>T</sup> and (F) *Lactobacillus rham-*

*nosus* DSM 20021<sup>T</sup>; 1, 4, 7, 10, 13, 16, *casei*/Y2; 2, 5, 8, 11, 14, 17, *para*/Y2; 3, 6, 9, 12, 15, 18, *rham*/Y2; 19, negative control; kb, linear DNA kb-ladder; pUC, pUC19-DNA/*Msp*I(*Hpa*II) marker.

Figure 3: Levels of yeasts (*Candida pulcherrima* 1-50/13) in 5 different batches of a food model with different protective cultures, stored at 6°C. (♦)  $4.9 \times 10^8$  cfu/g *Propionibacterium jensenii* SM11 and  $1.2 \times 10^8$  cfu/g *Lactobacillus paracasei* ssp. *paracasei* SM20; (▲)  $3.2 \times 10^8$  cfu/g *Propionibacterium jensenii* SM11 and  $1.1 \times 10^8$  cfu/g *Lactobacillus paracasei* ssp. *paracasei* SM29; (■)  $4.1 \times 10^8$  cfu/g *Propionibacterium jensenii* SM11 and  $9.1 \times 10^7$  cfu/g *Lactobacillus paracasei* subsp. *paracasei* SM63; (□)  $2.7 \times 10^8$  cfu/g *Propionibacterium jensenii* SM11 and  $2.1 \times 10^8$  cfu/g *Lactobacillus plantarum* SM17; (O)  $2.7 \times 10^8$  cfu/g *Propionibacterium jensenii* SM11 and  $9.3 \times 10^7$  cfu/g *Lactobacillus plantarum* SM39; (•) no protective culture. Number of yeasts are mean values of duplicates.

Figure 4: Levels of yeasts (*Candida pulcherrima* 1-50/13, *Candida magnoliae* 1-35/1, *Candida parapsilosis* 4-5/1 and *Zygosaccharomyces bailii* 1-48/1) in 7 different batches of yoghurt with different protective cultures stored at 6°C. (O)  $1.5 \times 10^7$  cfu/g *Propionibacterium jensenii* SM11 and  $4.3 \times 10^7$  cfu/g *Lactobacillus paracasei* ssp. *paracasei* SM20; (♦)  $5.5 \times 10^7$  cfu/g *Propionibacterium jensenii* SM11 and  $1.7 \times 10^8$  cfu/g *Lactobacillus paracasei* ssp. *paracasei* SM20; (□)  $2.0 \times 10^7$  cfu/g *Propionibacterium jensenii* SM11 and  $3.8 \times 10^7$  cfu/g *Lactobacillus paracasei* ssp. *paracasei* SM29; (■)  $2.8 \times 10^8$  cfu/g *Propionibacterium jensenii* SM11 and  $1.7 \times 10^8$  cfu/g *Lactobacillus paracasei* ssp. *paracasei* SM29; (Δ)  $1.6 \times 10^7$  cfu/g *Propionibacterium jensenii* SM11 and  $1.7 \times 10^7$  cfu/g *Lactobacillus paracasei* ssp. *paracasei* SM63; (▲)  $8.1 \times 10^7$  cfu/g *Propionibacterium jensenii* SM11 and  $1.6 \times 10^8$  cfu/g *Lactobacillus paracasei* ssp. *paracasei* SM63; (•) no protective culture. Number of yeasts are mean values of duplicates.

## Examples

### Material and Methods

#### Bacterial strains and media

[0093] Bacterial strains and fungi used in this study are listed in Table 1.

EP 1 308 506 A1

Table 1:

Microbial strains used in this study.				
Strain	Relevant characteristics	Culture conditions		Reference
		Temp (°C)	Medium	
Propionibacteria and lactobacilli				
Propionibacterium jensenii				
SM11	Antimicrobial activity	32	NLB	LME <sup>a</sup>
Lactobacillus casei				
DSM 20011 <sup>T</sup>	Type strain	30	MRS	DSM <sup>b</sup>
Lactobacillus paracasei				
subsp. paracasei				
SM20	Antimicrobial activity	32	MRS	This study
SM29	Antimicrobial activity	32	MRS	This study
SM63	Antimicrobial activity	32	MRS	This study
DSM 5622 <sup>T</sup>	Type strain	30	MRS	DSM
Lactobacillus plantarum				
SM17	Antimicrobial activity	32	MRS	This study
SM39	Antimicrobial activity	32	MRS	This study
Lactobacillus rhamnosus				
DSM 20021 <sup>T</sup>	Type strain	37	MRS	DSM
Other strains				
Bacillus cereus				
DSM 31 <sup>T</sup>	Type strain	30	BHI	DSM
Bacillus subtilis				
168		30	BHI	LME
Enterococcus faecalis				
DS5		37	BHI	LME
Staphylococcus aureus				
VF4		37	BHI	LME
Listeria innocua				
L17		37	BHI	LME
Listeria monocytogenes				
M1		37	BHI	LME
Citrobacter freundii				
SG84		30	BHI	LME
Enterobacter cloacae				
SG95		30	BHI	LME
Escherichia coli				
B		37	BHI	LME
SG63		37	BHI	LME



EP 1 308 506 A1

Table 1 (continued):

Microbial strains used in this study.				
Strain	Relevant characteristics	Culture conditions		Reference
<i>Klebsiella pneumoniae</i>				
SG89		30	BHI	LME
<i>Salmonella typhimurium</i>				
ATCC 14028		37	BHI	ATCC <sup>c</sup>
<i>Serratia liquefaciens</i>				
SG64		30	BHI	LME
<i>Candida</i> sp.				
1-50/15		25	YM	Miescher (1999)
<i>Candida krusei</i>				
3-69/2		25	YM	Miescher (1999)
<i>Candida magnoliae</i>				
1-35/1		25	YM	Miescher (1999)
<i>Candida parapsilosis</i>				
4-5/1		25	YM	Miescher (1999)
<i>Candida pulcherrima</i>				
1-50/13		25	YM	Miescher (1999)
<i>Candida reukaufii</i>				
4-73/4		25	YM	Miescher (1999)
<i>Candida silvicola</i>				
4-42/1		25	YM	Miescher (1999)
<i>Candida valida</i>				
1-48/8		25	YM	Miescher (1999)
<i>Sporobolomyces salmoni-</i> <i>color</i>				
3-46/2		25	YM	Miescher (1999)
<i>Zygosaccharomyces bailii</i>				
1-48/1		25	YM	Miescher (1999)
<i>Penicillium</i> sp.				
2-21/7		25	YM	Miescher (1999)
2-21/12		25	YM	Miescher (1999)
2-52/2		25	YM	Miescher (1999)
3-58/3		25	YM	Miescher (1999)

<sup>a</sup>Laboratory for Food Microbiology, Zurich, Switzerland

<sup>b</sup> German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany

<sup>c</sup> American Type Culture Collection, Manassas, VA, USA

<sup>T</sup> Type strain

[0094] *Propionibacterium* strains were grown in NLB broth that consisted of 1 % trypticase soy broth without dextrose (BBL Microbiology Systems, Cockeysville, MD, USA), 1 % yeast extract and 1 % sodium lactate syrup (Sigma Chemical Co., St. Louis, MO, USA) as described by Grinstead and Barefoot (1992). Lactobacilli were propagated in MRS broth

with 0.1 % Tween 80 (Biolife, Italy). All other strains were grown in BHI broth (Biolife, Italy) at growth conditions given in Table 1. Bacterial cultures were maintained as frozen stocks at -80°C in broth containing 30 % glycerol. Yeasts and moulds were cultured in YM medium (Difco, Detroit, MI, USA) and maintained at 4°C on solid agar medium.

[0095] Commercial starters for yoghurt fermentation were obtained from Danisco Cultor Niebüll GmbH, Germany, consisting of different strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*.

[0096] Protective cultures were produced by growing the strains separately in supplemented whey permeate (SWP1) that consisted of 5.5 % whey permeate (Emmi, Switzerland), 1 % yeast extract (Becton Dickinson, MD, USA) and 2 % casein hydrolysate (Merck, Germany) for 72 h at 32°C. The cells were harvested by centrifugation, washed once or twice in 0.85 % NaCl and resuspended in tyndallized skim milk (Difco, Detroit, MI, USA) or in 0.85 % NaCl in a necessary concentration ranging from 10- to 100-fold. Tyndallized skim milk was previously heat treated three times at 90°C for 30 min on three following days.

[0097] A scale up and a process optimisation was performed by growing the cells separately in a 8-1 or a 19-1 bioreactor (type L1523; Bioengineering, Switzerland), at 32°C, with an agitation of 250 rpm using 2 % inoculum. Fermentation was performed in supplemented whey permeate (SWP2) that consisted of 5.5 % whey permeate, 1 % yeast extract and 2 % glucose. The pH was controlled at 6.0 adding 3 M NaOH. Simultaneously with the pH control, a feed of nutrients was achieved with 20 % yeast extract and 20 % glucose. The inoculum was grown in supplemented whey permeate (SWP1) at 32°C for 72 h without agitation. Samples were withdrawn from the fermentations periodically and analysed for cell concentrations and optical density at 650 nm. Lactobacilli were grown for 72 h, propionibacteria for 90 h. The cells were harvested, washed and concentrated as described above.

#### Isolation of lactic acid bacteria

[0098] 10 g of food sample was stomached for 2 minutes with 90 ml of dilution-buffer (0.85 % NaCl, 0.1 % peptone), serially diluted and plated on MRS agar with 0.1 % Tween 80. Milk samples were directly diluted and plated. The plates were incubated under anaerobic conditions at 37°C for 3 days. From each food sample, a variety of different colonies was chosen for a screening for antimicrobial activities.

#### Screening methods for antimicrobial activities

[0099] Two methods were used:

(1) **Agar spot assay:** The producer culture was spot inoculated onto an agar plate according to Grinstead and Barefoot (1992) and incubated at optimal growth conditions (Table 1). Each plate was overlaid with 6 ml of tempered soft agar (0.6 %) containing 0.2 ml of an indicator culture which had grown up to a density of 0.2. The optical density of bacteria was measured at 650 nm, that of yeasts and moulds at 580 nm. Agar medium, soft agar medium and incubation of plates was chosen appropriate to the test and indicator organisms. Inhibition of the indicator lawn was monitored after 24 to 48 h for bacteria, fungi were daily scored for inhibition, after 3 days at 25°C.

(2) **Well diffusion test:** Wells of a diameter of 7 mm were cut to agar plates and filled with cell-free supernatants of bacterial cultures as described by Daeschel (1992). After complete diffusion of the liquid, soft agar containing an indicator organism was poured over the plates to demonstrate inhibitory activity as described above. Agar medium, soft agar medium and incubation of plates was chosen appropriate to the test and indicator organisms.

#### Characterisation and identification of bacterial isolates

[0100] Isolates grown on MRS agar showing antimicrobial activities were Gram-stained and examined microscopically for cellular morphology. Furthermore catalase activity was tested by spotting colonies with 30 % hydrogen peroxide and a staining of spores was done. A preliminary strain identification was done using API 50 CHL (bioMérieux SA, Marcy l'Etoile, France). Additionally, proteolysis was determined in MRS agar with 5 % and 10 % skim milk and production of organic acids was analysed enzymatically (Boehringer Mannheim, Germany; kit used for D/L-lactic acid: E1 112 821 / kit used for acetic acid: E0 148 261). Furthermore, growth conditions were determined in MRS broth at different temperatures for 72 h and with salinities of 5 % and 10 % at 32°C for 72 h.

#### Identification of selected strains by partial 16S rDNA sequencing

[0101] Amplification of 16S rDNA was carried out using directly a colony of the corresponding strain. PCR was performed with universal oligonucleotides bak4 (Goldenberger, 1997) and bak11w (Greisen et al., 1994 modified by Dasen et al. 1998) using 40 cycles. Annealing was achieved at 56°C for 30 sec and extension occurred with Taq DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) at 72°C for 2 min, DNA strand separation was performed at

# EP 1 308 506 A1

95°C for 5 sec. Amplified DNA was purified using the GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). Automated cycle sequencing was done with the Terminator Ready Reaction Mix (Amersham Pharmacia Biotech, Uppsala, Sweden) and oligonucleotides targeting the 16S rDNA (Table 2).

5

10

15

20

25

30

35

40

45

50

55

Table 2: Oligonucleotides targeting the 16S rDNA.

Name	Sequence	Comment	Reference
Y2	5'-TGGCTCAGAACGACGCTAGGCCG-3'	Conserved 16S rDNA <sup>a</sup>	Young <i>et al.</i> (1991)
<i>casei</i>	5'-TGCACGTGAGATTCGACTTAA-3'	<i>Lactobacillus casei</i> 16S rDNA	Ward and Timmins (1999)
<i>para</i>	5'-CACCGAGATTCAACATGG-3'	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> 16S rDNA	Ward and Timmins (1999)
<i>ram</i>	5'-TGCAJCTTGTATTTAATTTTG-3'	<i>Lactobacillus rhamnosus</i> 16S rDNA	Ward and Timmins (1999)
bak4	5'-AGGAGGTGATCCARCCGCA-3'	Conserved 16S rDNA	Greisen <i>et al.</i> (1994)
bak11w	5'-AGTTTGATCMTGGCTCAG-3'	Conserved 16S rDNA	Goldenberger (1997)
eub338	5'-ACTCCTACGGGAGGCAGC-3'	Conserved 16S rDNA	Amann <i>et al.</i> (1995)
uni515	5'-ACCGGGCTGCTGGCAC-3'	Conserved 16S rDNA	Lane (1991)
uni785	5'-GGMTTAGATACCCCTGGTAGTCC-3'	Conserved 16S rDNA	Amann <i>et al.</i> (1995)
uni1088	5'-CGTTAAGTCCCGCAACGAGC-3'	Conserved 16S rDNA	Amann <i>et al.</i> (1995)
uni1392	5'-GTACACACCGCCCGTCA-3'	Conserved 16S rDNA	Lane (1991)

<sup>a</sup> corresponding to a conserved region of most of the known bacterial 16S rDNA sequences

[0102] 35 cycles were performed as follows: 96°C for 10 sec, 50°C for 30 sec, 60°C for 4 min. Nucleotide sequencing

of both strands from cloned DNA was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) with primer walking using the BigDye Terminator Cycle Sequencing Ready Reaction Kit and the ABI PRISM ABI 310 Genetic Analyser apparatus (Applied Biosystems, Foster City, CA, USA) for analysis. DNA sequence analysis, sequence alignments, and sequence database searching were conducted with programs contained within the Sequence Analysis Software Package (version 10.0) licensed from the Genetics Computer Group (University of Wisconsin, Madison, WI, USA).

[0103] Sequences were compared by the algorithm of Pearson and Lipman (1988) (FastA and TFastA) with sequences in the GenEMBL database copy.

#### 10 Differentiation of *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction

[0104] Amplification of 16S rDNA was carried out as described by Ward and Timmins (1999) with some modifications. A 290-bp fragment of the 16S rDNA was amplified using species-specific primers (*casei*, *para*, *rham*) in conjunction with primer Y2 corresponding to a conserved region of the 16S rDNA (Table 2). Each 50- $\mu$ l reaction contained 2.5 U of Taq polymerase, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, 1 mM dNTP Set, 1  $\mu$ M each primer and one colony of the corresponding strain. Taq polymerase, buffers and dNTP Set for PCR were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Oligonucleotides were synthesised by Microsynth (Balgach, Switzerland). The temperature program was as described by Young *et al.* (1991): 300s at 93°C, 35 cycles of (45s at 93°C, 45 s at 62°C and 120 s at 72°C) and 300 s at 72°C with a final cooling to 4°C. Amplification products were separated on a 2 % agarose gel.

#### Investigation of antimicrobial activities in agar plates

[0105] Agar plates were prepared, supplemented with *Propionibacterium jensenii* SM11 or an antimicrobial *Lactobacillus* or a combination of both. The cultures were previously grown separately for 72 h at 32 °C in supplemented whey permeate (SWP1), centrifugated, washed once and resuspended (1:1) in 0.85% NaCl and then serially diluted in dilution-buffer (0.85 % NaCl, 0.1 % peptone). Portions of 20 ml of agar were prepared with 10% of each dilution to give a final level of 10<sup>5</sup>, 10<sup>6</sup>, 10<sup>7</sup> and 10<sup>8</sup> cfu/ml. Agar plates were poured and after solidifying, portions of approximately 2-5  $\mu$ l of broth cultures of the indicator strains were spot inoculated on the plates. YM agar was used for yeasts and moulds and BHI agar for bacteria. Triplex of the agar plates were stored at 6°C, 12°C and 25°C for up to 4 weeks and the growth of indicator organisms was controlled weekly for 6°C or daily for 12°C and 25°C. As a control, agar plates without supplementation of lactobacilli and propionibacteria were prepared as well as agar plates with an addition of 1g/l Ka-Sorbat (EU-Richtlinie, Nr. 95/2/EG).

#### Investigation of antifungal activities in a food model

[0106] A food model was set up with portions of 50 g tyndallized skim milk (Difco, Detroit, MI, USA) that were fermented with a commercial yoghurt culture. Test samples were additionally inoculated with a protective culture (1 % of a 100-fold concentrated culture of *Propionibacterium jensenii* SM11 and 1 % of a 100-fold concentrated culture of an antifungal *Lactobacillus*) to give a level of 10<sup>8</sup> cells/g of each. The cultures were previously washed twice and resuspended in tyndallized skim milk. To get comparable results, the control sample was mixed with 2 % tyndallized skim milk. The fermentation was done for about 5 hours at 42 °C until the pH reached 4.6. All samples were contaminated with *Candida pulcherrima* 1-50/13 at a final level of 10<sup>2</sup> cfu/g. Additionally a control sample was prepared without a yeasts contamination. Samples were stored at 6°C for 7 weeks and protective propionibacteria and lactobacilli as well as yeasts were enumerated weekly. Simultaneously, pH was controlled weekly.

#### Inhibition of fungi in yoghurt by protective cultures

[0107] Portions of 200 g milk enriched with 5% cream (35 % fat, 2.5 % proteins), 2 % skim milk powder and 5 % sugar were heat treated at 90°C for 10 min and then fermented with a commercial yoghurt culture. Test samples were additionally inoculated with a protective culture (0.1 % or 1 % of a 100-fold concentrated culture of *Propionibacterium jensenii* SM11 and 0.1 % or 1 % of a 100-fold concentrated culture of an antifungal *Lactobacillus*-strain) to give a level of 10<sup>7</sup> or 10<sup>8</sup> cfu/g of each. Protective cultures were previously washed twice and resuspended in tyndallized skim milk. The fermentation was done for about 5 hours at 42 °C until the pH reached 4.6. The yoghurt was contaminated with a mix of *Candida pulcherrima* 1-50/13, *Candida magnoliae* 1-35/1, *Candida parapsilosis* 4-5/1 and *Zygosaccharomyces bailii* 1-48/1 at a final level of 10<sup>2</sup> cfu/g. Additionally a control sample was prepared without a yeasts contamination. The yoghurt was stored at 6°C for 4 weeks and yeasts were enumerated weekly. Simultaneously, pH was

controlled weekly. At day 1 and after 3 weeks of storage, protective propionibacteria and lactobacilli were enumerated and furthermore after 3 weeks lactate and acetate were determined enzymatically (Boehringer Mannheim, Germany; kit used for D/L-lactic acid: E1 112 821 / kit used for acetic acid: E0 148 261).

#### 5 Scale up of the application of a protective culture in yoghurt

[0108] Scale up trials with yoghurt comprising the protective culture were also performed as described above.

#### Inhibition of yeasts on a cheese surface by protective cultures

10

[0109] Equal cubes (2.5 x 4.8 x 8 cm) of 100 g hard cheese (Gruyère) were prepared. To avoid a contamination with microorganisms of the cheese surface, 3-5 mm of the cheese's rind were previously cut off. One batch of cheese was soaked with each cube's side in a protective culture (5 ml of a 20-fold concentrated culture of *Propionibacterium jensenii* SM11 and 5 ml of a 20-fold concentrated culture of an antifungal *Lactobacillus-strain*) to give a level of  $10^8$  cfu/g cheese surface of each. Protective cultures were previously washed twice and resuspended in 0.85 % NaCl. Two other batches of cheese were prepared with 10-fold and 100-fold diluted protective cultures. Onto the surface of the cubes of all three batches, about 3 ml of the respective concentrated protective culture was thus applied. Then, the cheeses were contaminated with 1 ml ( $10^4$  cfu/ml) of a mix of *Candida pulcherrima* 1-50/13, *Candida magnoliae* 1-35/1, *Candida parapsilosis* 4-5/1 and *Zygosaccharomyces bailii* 1-48/1 to reach a final level of  $10^2$  cfu/g cheese surface. The surface of the cheeses was defined as a layer of about 3 mm from the outside of the cube. A control sample was prepared without an addition of yeasts. The cheeses were sealed in sterile plastic bags and stored for 3 weeks at 6°C. At day 1 and after 3 weeks of storage, the level of protective propionibacteria and lactobacilli was determined as well as the number of yeasts.

15

20

25

#### Inhibition of *Listeria* by a protective culture in a food model

[0110] Two food models were set up to test antibacterial activities of a protective culture against *Listeria*.

##### (1) Inhibition of *Listeria innocua* L17 and *Listeria monocytogenes* M1 by a protective culture in cream:

30

[0111] 200 g of pasteurised half-cream (25 % fat, 2.5 % proteins) were inoculated with *Listeria innocua* L17 or *Listeria monocytogenes* M1, respectively, at a final level of  $10^3$  cfu/g and were then divided into two portions of 100 g in sterile flasks. One sample was inoculated with a protective culture (5 % of a 20-fold concentrated culture of *Propionibacterium jensenii* SM11 and 5 % of a 20-fold concentrated culture of *Lactobacillus paracasei* subsp. *paracasei* SM20) to give a level of  $10^8$  cfu/g of each. Protective cultures were previously washed twice and resuspended in tyndallized skim milk. To get comparable concentrations, 10 % of tyndallized skim milk were added to the control sample. All samples were stored at 6 °C for 4 weeks. The pH and growth of *Listeria* was controlled weekly, after 1 day, and after 4 weeks the level of protective culture was checked as well. A second batch was prepared as described and stored at 25°C for 3 weeks. Similarly, the numbers of protective cultures and of *Listeria* were determined at the beginning and at the end of storage.

35

40

##### (2) Inhibition of *Listeria innocua* L17 by protective cultures in minced meat:

[0112] Portions of 100 g of minced meat were mixed with a protective culture (5 % of a 20-fold concentrated culture of *Propionibacterium jensenii* SM11 and 5 % of a 20-fold concentrated culture of an antimicrobial *Lactobacillus-strain*) to give a level of  $10^8$  cfu/g of each. Protective cultures were previously washed twice and resuspended in 0.85 % NaCl. The meat was contaminated with *Listeria innocua* L17 at a final concentration of  $10^3$  cfu/g and was then stored at 6°C for 8 weeks. Additionally a control sample was prepared without protective culture. To get comparable concentrations, 10 % of 0.85 % NaCl were added to this sample. Protective propionibacteria and lactobacilli as well as *Listeria innocua* L17 were enumerated weekly, pH was controlled at the end of the storage period.

45

50

## Results

### Antifungal activities in *Lactobacillus* sp.

55

[0113] A preliminary screening for antimicrobial activities was performed with 1424 isolates on MRS agar originating from raw milk, cheese, yoghurt, black olives, sour dough, salami, as well as maize and gras silage. A total of 82 strains showed activities in an agar spot assay against the fungi listed in Table 1. These isolates were further characterised

by Gram-staining, catalase-activity, microscopy and forming of spores. As depicted in Figure 1 the species belonging to the *Lactobacillus casei*-group showed predominantly antifungal activities with a share of 24 %. A preliminary identification with API 50 CHL classified these isolates as *Lactobacillus casei*, *Lactobacillus paracasei* subsp. *paracasei* and *Lactobacillus rhamnosus*. Furthermore the species *Lactobacillus plantarum* showed high inhibitory activities towards the tested fungi. A total of 6 % of the antifungal isolates appeared to be cocci and 18 % remained unidentified using fermentation patterns.

#### Identification and characterisation of three high antifungal lactobacilli

[0114] Three isolates with high antifungal activities were further identified using sequencing analysis of the 16S rDNA. Studying carbohydrate metabolisms, they were previously identified as *Lactobacillus paracasei* subsp. *paracasei* (SM20 and SM29) and *Lactobacillus curvatus* (SM63). Sequencing analysis of the 16S rDNA of these strains revealed the species *Lactobacillus paracasei* subsp. *paracasei* for strain SM20 with an identity of 100 % in 1519 nucleotides overlapping with the partial sequence of the 16S rDNA of *Lactobacillus paracasei* JCM8130 (D79212) and with the partial 16S rDNA-sequence of a *Lactobacillus casei* (D86517). A total of 1520 nucleotides were identified from the 16S rDNA of strain SM20. 1528 nucleotides sequenced from strain SM29 showed an identity of 99.9 % in 1520 nucleotides overlapping with the partial sequence of the 16S rRNA gene of a *Lactobacillus casei* (D86517) and with the partial 16S rDNA-sequence of *Lactobacillus paracasei* JCM8130 (D79212). Surprisingly, 1515 nucleotides identified of the 16S rRNA gene of strain SM63 showed also an identity of 99.9 % in 1493 nucleotides overlapping with the 16S rRNA gene of a *Lactobacillus casei* (D86517) and with the partial sequence of the 16S rDNA of *Lactobacillus paracasei* JCM8130 (D79212).

[0115] Since members of the casei-group form a very homologous group concerning phenotypic criteria as well as sequence data of the 16S rRNA gene, Ward and Timmins (1999) developed a simple PCR approach to differentiate the species *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* based on the reclassification by Collins *et al.* (1989). Figure 2 shows a specific product of 290 bp for strains SM20, SM29 and SM63 amplified in a polymerase chain reaction with the *Lactobacillus paracasei*-specific primer used in conjunction with primer Y2, both targeting the 16S rRNA gene. The type strains of *Lactobacillus casei* (DSM 20011<sup>T</sup>) and *Lactobacillus paracasei* subsp. *paracasei* (DSM 5622<sup>T</sup>) also revealed a 290-bp band with the corresponding primers. Surprisingly, *Lactobacillus rhamnosus* (DSM 20021<sup>T</sup>) did not show the specific band after polymerase chain reaction with Y2 and *rham*. Furthermore, no products were observed if this primer pair (Y2/*rham*) was used with the strains to be identified. Thus, the isolates SM20, SM29 and SM63 were identified as *Lactobacillus paracasei* subsp. *paracasei*. Table 3 shows a summary of the identification of the three isolates as well as their characterisation.

Table 3: Identification and characterisation of strains SM20, SM29, SM63 and SM11.

Strain	Origin	Microscopy	Spores	Acid produced from <sup>a</sup>	Fermentation products <sup>b</sup>	Growth conditions	NaCl Tolerance	Proteolytic activity	Catalase activity	API 50 CHL	Identification Sequencing <sup>c</sup>	PCR <sup>d</sup>
SM20	Raw milk	short rods in chains, Gram-positive <sup>e</sup>	negative	Ribose, Galactose, D-Glucose, D-Fructose, D-Mannose, Mannitol, Sorbitol, N Acetyl glucosamine, Amygdaline, Arbutine, Esculine, Salicine, Cellobiose, Maltose, Lactose, Saccharose, Trehalose, Inuline, Melezitose, $\beta$ Gentiobiose, D-Turanose, D-Tagatose	D-, L-Lactate	15-45°C (Opt: 32-37°C)	10% <sup>g</sup>	negative	negative	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> and <i>Lactobacillus casei</i>	<i>Lactobacillus casei</i> subsp. <i>paracasei</i>
SM29	Salami	short rods in chains, Gram-positive	negative	Ribose, Adonitol, Galactose, D-Glucose, D-Fructose, D-Mannose, Rhamnose, Dulcitol, Mannitol, Sorbitol, $\alpha$ Methyl-D-mannoside, $\alpha$ Methyl-D-glucoside, N Acetyl glucosamine, Amygdaline, Arbutine, Esculine, Salicine, Cellobiose, Maltose, Lactose, Saccharose, Trehalose, Inuline, Melezitose, $\beta$ Gentiobiose, D-Turanose, D-Tagatose	D-, L-Lactate	15-45°C (Opt: 32-37°C)	5% <sup>h</sup>	negative	negative	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> and <i>Lactobacillus casei</i>	<i>Lactobacillus casei</i> subsp. <i>paracasei</i>



Table 3 (continued)

Strain	Origin	Microscopy	Spores	Acid produced from <sup>a</sup>	Fermentation products <sup>b</sup>	Growth conditions	NaCl Tolerance	Proteolytic activity	Catalase activity	API 50 CHL	Identification <sup>c</sup>	PCR <sup>d</sup>
SM63	Raw milk	short rods in chains, Gram-positive	negative	Ribose, Galactose, D-Glucose, D-Fructose, D-Mannose, Mannitol, Sorbitol, N Acetyl glucosamine, Maltose, Lactose, Trehalose, Melezitose, D-Tagatose	D-, L-Lactate	15-45°C (Opt: 32-37°C)	5% <sup>i</sup>	negative	negative	<i>Lactobacillus curvatus</i>	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> and <i>Lactobacillus casei</i>	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
SM11	Raw milk	pleomorphic rod-shaped, Gram-positive		Erythritol, D-Arabinose, Ribose, Adonitol, Galactose, D-Glucose, D-Fructose, D-Mannose, Lactose, D-Arabinol,	Acetate, Propionate <sup>j</sup>	15-37°C (Opt: 30-32°C)	5% <sup>k</sup>	negative	positive		<i>Propionibacterium jensenii</i>	

<sup>a</sup> determined using API 50 CHL<sup>b</sup> determined enzymatically (Boehringer Mannheim, Germany; kit used for D/L-lactic acid: E1 112 821 / kit used for acetic acid: E0 148 261)<sup>c</sup> sequencing analysis of the 16S rDNA<sup>d</sup> PCR approach modified according to Ward and Timmins (1999)<sup>e</sup> long rods in chains in bioreactor cultures<sup>f</sup> Miescher (1999)<sup>g</sup> well growth at a salinity of 5 % and fairly well growth at 10 %<sup>h</sup> well growth at a salinity of 5 % NaCl and very weak growth at 10 %<sup>i</sup> well growth at a salinity of 5% and weak growth at 10 %<sup>k</sup> fairly well growth at a salinity of 5 % NaCl and very weak growth at 10 %<sup>l</sup> underlined species indicates the final identification of the corresponding strain[0116] Collins et al. (1989) described another subspecies of *Lactobacillus paracasei*, namely *Lactobacillus paracasei*

subsp. *tolerans*. Regarding the acid production observed by Collins *et al.* (1989), the isolates SM20, SM29 and SM63 belong to the subspecies *paracasei*.

#### Secretion of inhibitory substances into broth medium

[0117] *Lactobacillus paracasei* subsp. *paracasei* SM20, *Lactobacillus paracasei* subsp. *paracasei* SM29, *Lactobacillus paracasei* subsp. *paracasei* SM63, *Lactobacillus plantarum* SM17 and *Lactobacillus plantarum* SM39 were examined for their ability to secrete inhibitory substances into broth medium. Therefore strains were either grown in MRS broth or in SWP1 medium. No antagonistic activity was detected in the cell-free supernatants using a well diffusion test. Even a concentration step by ultrafiltration (molecular weight cut-off of 3'000 Da) did not reveal any detectable activity (data not shown).

#### Antifungal activities in agar plates

[0118] A total of 18 lactobacilli were tested for their antifungal activities alone or in combination with the high-antifungal *Propionibacterium jensenii* SM11. Therefore, the strains were incorporated, after centrifugation and a washing step in concentrations from  $10^5$  to  $10^8$  cfu/ml in YM agar plates on which the fungi listed in Table 1 were spotted in corresponding levels from  $10^1$  to  $10^7$  cfu/ml. The plates were stored in different batches at 6°C, 12°C and 25°C. The growth of yeasts was controlled weekly or daily, respectively. Although, the lactobacilli as well as the *Propionibacterium* strain showed only weak inhibitory activities using them alone (data not shown) their combination revealed high antagonistic values at 6°C. The highest activities were detected with the following lactobacilli in combination with *Propionibacterium jensenii* SM11: *Lactobacillus paracasei* subsp. *paracasei* SM20, *Lactobacillus paracasei* subsp. *paracasei* SM29 and *Lactobacillus paracasei* subsp. *paracasei* SM63. Weak activities were observed with *Lactobacillus plantarum* SM17 and *Lactobacillus plantarum* SM39 in conjunction with strain SM11. A cell number of  $10^8$  cfu/ml of the *Lactobacillus*-strain and  $10^8$  cfu/ml of the *Propionibacterium*-strain was necessary for high inhibitory activities. Table 4 indicates five examples of the antifungal activities observed at 6°C with a concentration of  $10^8$  cfu/ml ( $10^7$  cfu/ml for *Lactobacillus plantarum* SM17) against spot-inoculated yeasts in a density of  $10^6$  cfu/ml and against spot-inoculated 3-day cultures of moulds.

Table 4: Antifungal activities of selected protective cultures incorporated in agar plates  
after a storage of 21 days at 6°C, or 7 days at 12°C.

Indicator organisms <sup>a</sup>	Protective cultures <sup>b</sup>						
	SM20/SM11 <sup>c</sup> (pH 3.83)	SM29/SM11 (pH 3.98)	SM63/SM11 (pH 3.93)	SM17/SM11 (pH 4.53)	SM39/SM11 (pH 4.59)	control (pH 5.88)	Ka-Sorbat (lg/l) <sup>d</sup>
Storage at 6°C / 21 d							
<i>Candida magnoliae</i> 1-35/1	+	+/-	+	++	++	++	++
<i>Candida parapsilosis</i> 4-5/1	-	-	-	-	-	(+/-)	-
<i>Zygosacharomyces bailii</i> 1-48/1	-	-	-	-	-	-	-
<i>Candida silvicola</i> 4-42/1	(+/-)	(+/-)	(+/-)	++	++	++	++
<i>Candida valida</i> 1-48/8	(+/-)	(+/-)	(+/-)	++	++	+	-
<i>Candida pulcherrima</i> 1-50/13	(+/-)	-	(+/-)	++	++	++	++
<i>Candida reukauffii</i> 4-73/4	-	-	-	++	++	++	++
<i>Candida</i> sp. 1-50/15	(+/-)	(+/-)	(+/-)	++	++	++	++
<i>Sporobolomyces salmonicolor</i> 3-46/2	-	-	-	-	-	++	-
<i>Candida krusei</i> 3-69/2	-	-	-	(+/-)	(+/-)	(+/-)	-
<i>Penicillium</i> sp. 2-21/7	-	-	-	n.d.	n.d.	++	n.d.
<i>Penicillium</i> sp. 2-21/12	-	-	-	n.d.	n.d.	++	n.d.
<i>Penicillium</i> sp. 2-52/2	-	-	-	n.d.	n.d.	++	n.d.
<i>Penicillium</i> sp. 3-58/3	-	-	-	n.d.	n.d.	++	n.d.

Table 4 (continued)

Indicator organisms <sup>a</sup>	Protective cultures <sup>b</sup>						K.a-Sorbat (1g/l) <sup>d</sup>
	SM20/SM11 (pH 3.83) <sup>c</sup>	SM29/SM11 (pH 3.98)	SM63/SM11 (pH 3.93)	SM17/SM11 (pH 4.53)	SM39/SM11 (pH 4.59)	control (pH 5.88)	
Storage at 12°C / 7 d							
<i>Candida magnoliae</i> 1-35/1	(+/-)	+	+	+	+	++	n.d
<i>Candida parapsilosis</i> 4-5/1	-	(+/-)	-	+/-	+/-	++	n.d
<i>Zygosacharomyces bailii</i> 1-48/1	-	(+/-)	(+/-)	(+/-)	-	+/-	n.d
<i>Candida silvicola</i> 4-42/1	(+/-)	(+/-)	(+/-)	(+/-)	(+/-)	++	n.d
<i>Candida valida</i> 1-48/8	-	+	+	++	+	++	n.d
<i>Candida pulcherrima</i> 1-50/13	-	++	+	++	++	++	n.d
<i>Candida reukaufii</i> 4-73/4	-	+	-	++	++	++	n.d
<i>Candida</i> sp. 1-50/15	-	++	++	++	++	++	n.d
<i>Sporobolomyces salmonicolor</i> 3-46/2	-	-	-	+	+/-	+/-	n.d
<i>Candida krusei</i> 3-69/2	+	++	++	++	++	++	n.d

SM20: *Lactobacillus paracasei* subsp. *paracasei* SM20 (10<sup>8</sup> cfu/ml)SM29: *Lactobacillus paracasei* subsp. *paracasei* SM29 (10<sup>8</sup> cfu/ml)SM63: *Lactobacillus paracasei* subsp. *paracasei* SM63 (10<sup>8</sup> cfu/ml)SM17: *Lactobacillus plantarum* SM17 (10<sup>7</sup> cfu/ml)SM39: *Lactobacillus plantarum* SM39 (10<sup>7</sup> cfu/ml)SM11: *Propionibacterium jensenii* SM11 (10<sup>8</sup> cfu/ml)

n.d.: not determined

<sup>a</sup> spot-inoculation with 10<sup>6</sup> cfu/ml<sup>b</sup> incorporated in agar plates<sup>c</sup> pH measured on agar plates after a storage of 21 days at 6°C<sup>d</sup> concentration according to EU-Richtlinie, Nr. 95/2/EG

++ strong colony (≈ 0% inhibition)

+ normal colony (≈ 25% inhibition)

+/- weak colony (≈ 50% inhibition)

(+/+) very weak colony (≈ 75% inhibition)

- no colony (≈ 100% inhibition)

[0119] Interestingly, using Ka-Sorbat, a weaker inhibitory activity was observed at 6°C than with the antimicrobial

cultures of this study (strains SM20, SM29 or SM63 in combination with strain SM11). Table 4 further depicts, that after a storage of 7 days at 12°C a clear inhibitory activity was observed for *Lactobacillus paracasei* subsp. *paracasei* strains SM20, SM29 and SM63 and a weak for *Lactobacillus plantarum* strains SM17 and SM39 in conjunction with *Propionibacterium jensenii* SM11. At 25°C only weak inhibitory activities were observed that disappeared after a few days (data not shown). Testing the antifungal activities of 2 or 3 lactobacilli (*Lactobacillus paracasei* subsp. *paracasei* strains SM20, SM29 and SM63) in conjunction with *Propionibacterium jensenii* SM11, an increase in inhibition was observed (Table 5) in comparison with the trials in which 1 *Lactobacillus* strain in combination with SM11 was used (Table 4). A total inhibition of the yeasts was reached using *Lactobacillus paracasei* subsp. *paracasei* strains SM20, SM29 or SM63 in conjunction with *Propionibacterium jensenii* SM11 and 1 g/l Ka-Sorbat (Table 5). The antifungal activity of the bacterial strains was increased with the food preservative.

Table 5: Antifungal activities of selected protective cultures incorporated in agar plates after a storage of 21 days at 6°C.

Indicator organisms <sup>a</sup>	Protective cultures <sup>b</sup>						
	SM20/SM29/ SM11 (pH 4.27) <sup>c</sup>	SM20/SM63/ SM11 (pH 4.32)	SM29/SM63/ SM11 (pH 4.33)	SM20/SM29/SM63/ SM11 (pH 4.10)	SM20/SM11/ Ka-Sorbat <sup>d</sup> (pH 4.67)	SM29/SM11/ Ka-Sorbat (pH 4.80)	SM63/SM11/ Ka-Sorbat (pH 4.75)
<i>Candida magnoliae</i> 1-35/1	(+/-)	(+/-)	+/-	(+/-)	-	-	++
<i>Candida parapsilosis</i> 4-5/1	-	-	-	-	-	-	-
<i>Zygosacharomyces bailii</i> 1-48/1	-	-	-	-	-	-	-
<i>Candida silvicola</i> 4-42/1	-	-	-	-	-	-	++
<i>Candida valida</i> 1-48/8	-	-	(+/-)	-	-	-	(+/-)
<i>Candida pulcherrima</i> 1-50/13	-	-	(+/-)	-	-	-	++
<i>Candida reukaufii</i> 4-73/4	-	-	-	-	-	-	++
<i>Candida</i> sp. 1-50/15	-	-	(+/-)	-	-	-	+
<i>Sporobolomyces salmonicolor</i> 3-46/2	-	-	-	-	-	-	+/-
<i>Candida krusei</i> 3-69/2	-	-	-	-	-	-	-

SM20: *Lactobacillus paracasei* subsp. *paracasei* SM20 (10<sup>8</sup> cfu/ml)SM29: *Lactobacillus paracasei* subsp. *paracasei* SM29 (10<sup>8</sup> cfu/ml)SM63: *Lactobacillus paracasei* subsp. *paracasei* SM63 (10<sup>8</sup> cfu/ml)SM11: *Propionibacterium jensenii* SM11 (10<sup>8</sup> cfu/ml)

n.d.: not determined

<sup>a</sup> spot-inoculation with 10<sup>6</sup> cfu/ml<sup>b</sup> incorporated in agar plates<sup>c</sup> pH measured on agar plates after a storage of 21 days at 6°C<sup>d</sup> 1 g/l, concentration according to EU-Richtlinie, Nr. 95/2/EG

++ strong colony (= 0% inhibition)

+ normal colony (= 25% inhibition)

+/- weak colony (= 50% inhibition)

( +/- ) very weak colony (= 75% inhibition)

. no colony (= 100% inhibition)

# Antibacterial activities of protective cultures in agar plates

[0120] *Lactobacillus paracasei* subsp. *paracasei* strains SM20, SM29 and SM63 were further tested for their ability to inhibit Gram-positive and Gram-negative bacteria alone or in combination with *Propionibacterium jensenii* SM11. Therefore, the strains were incorporated after centrifugation and a washing step in a concentration of  $10^8$  cfu/ml in BHI agar plates on which overnight cultures of the corresponding bacteria were spotted. One batch of the plates was stored at 6°C and the growth of bacteria was controlled weekly, a second was incubated at 25°C and was daily scored for inhibition. Again, the lactobacilli as well as the *Propionibacterium* strain showed weak inhibitory activities using them alone (data not shown) but their combination revealed high antagonistic values (Table 6).

EP 1 308 506 A1

Table 6:

Antibacterial activities of selected protective cultures incorporated in agar plates after a storage of 21 days at 6°C or at 25°C.					
5	Indicatororganisms <sup>a</sup>	Protective cultures <sup>b</sup>			contro1 (pH 6.70)
		SM20/SM11 (pH 5.45 °)	SM29/SM11 (pH 5.48)	SM63/SM11 (pH 5.40)	
Storage at 6°C / 21 d					
10	<i>Listeria innocua</i> L17	-	-	-	++
	<i>Staphylococcus aureus</i> VF4	-	-	-	(+/-)
	<i>Escherichia coli</i> B	-	-	-	(+/-)
15	<i>Escherichia coli</i> SG63	-	-	-	+/-
	<i>Sereratia liquefaciens</i> SG64	-	(+/-)	+	++
	<i>Citrobacter freundii</i> SG84	(+/-)	(+/-)	(+/-)	++
20	<i>Klebsiella pneumoniae</i> SG89	(+/-)	(+/-)	+/-	++
	<i>Enterobacter cloacae</i> SG95	(+/-)	(+/-)	(+/-)	+
25	<i>Enterococcus faecalis</i> DS5	-	-	-	+
	<i>Bacillus subtilis</i> 168	-	-	-	-
	<i>Bacillus cereus</i> DSM 31 <sup>T</sup>	-	-	-	-
30	<i>Salmonella typhimurium</i> ATCC 14058	-	-	-	++
	<i>Listeria moriocytozenes</i> M1	-	-	-	++
35	Storage at 25°C / 21 d				
	<i>Listeria innocua</i> L17	-	-	-	++
	<i>Staphylococcus aureus</i> VF4	+	+/-	+ /-	++
40	<i>Escherichia coli</i> B	+	(+/-)	(+/-)	++
	<i>Escherichia coli</i> SG63	+/-	-	-	++
	<i>Sereratia liquefaciens</i> SG64	+/-	+	+/-	++
45					

SM20: *Lactobacillus paracasei* subsp. *paracasei*

SM20 (10<sup>8</sup> cfu/ml)

SM29: *Lactobacillus paracasei* subsp. *paracasei*

SM29 (10<sup>8</sup> cfu/ml)

SM63: *Lactobacillus paracasei* subsp. *paracasei*

SM63 (10<sup>8</sup> cfu/ml) SM11: *Protonibacterium jensenii* SM11 (10<sup>8</sup> cfu/ml)

<sup>a</sup> spot-inoculation with an overnight culture

<sup>b</sup> incorporated in agar plates

<sup>c</sup> pH measured on agar plates after a storage of 21 days at 6°C

++ strong colony (= 0% inhibition)

+ normal colony (= 25% inhibition)

+/- weak colony (= 50% inhibition)

(+/-) very weak colony (= 75% inhibition) - no colony (= 100% inhibition)



# EP 1 308 506 A1

Table 6 (continued):

Antibacterial activities of selected protective cultures incorporated in agar plates after a storage of 21 days at 6°C or at 25°C.				
Indicatororganisms <sup>a</sup>	<u>Protective cultures<sup>b</sup></u>			
Storage at 25°C / 21 d				
<i>Citrobacter freundii</i> SG84	(+/-)	+/-	(+/-)	++
<i>Klebsiella pneumoniae</i> SG89	(+/-)	+/-	+	++
<i>Enterobacter cloacae</i> SG95	(+/-)	+	+	++
<i>Enterococcus faecalis</i> DS5	(+/-)	(+/-)	(+/-)	++
<i>Bacillus subtilis</i> 168	-	-	-	++
<i>Bacillus cereus</i> DSM 31 <sup>T</sup>	-	(+/-)	(+/-)	++
<i>Salmonella typhimurium</i> ATCC 14028	-	-	-	++
<i>Listeria monocytogenes</i> M1	-	-	-	++

SM20: *Lactobacillus paracasei* subsp. *paracasei*

SM20 (10<sup>8</sup> cfu/ml)

SM29: *Lactobacillus paracasei* subsp. *paracasei*

SM29 (10<sup>8</sup> cfu/ml)

SM63: *Lactobacillus paracasei* subsp. *paracasei*

SM63 (10<sup>8</sup> cfu/ml) SM11: *Propionibacterium jensenii* SM11 (10<sup>8</sup> cfu/ml)

<sup>a</sup> spot-inoculation with an overnight culture

<sup>b</sup> incorporated in agar plates

<sup>c</sup> pH measured on agar plates after a storage of 21 days at 6°C

++ strong colony (= 0% inhibition)

+ normal colony (= 25% inhibition)

+/- weak colony (= 50% inhibition)

(+/-) very weak colony (= 75% inhibition) - no colony (= 100% inhibition)

## Antifungal activities of stored protective cultures

[0121] In order to determine the ability to store the protective cultures, they were held separately at a 100-fold concentration (resuspended in tydallized skim milk) at - 80°C for 15 weeks. Then, the strains were incorporated in a concentration of 10<sup>8</sup> cfu/ml in YM agar plates on which the yeasts listed in Table 1 were spotted in levels from 10<sup>4</sup> to 10<sup>7</sup> cfu/ml. The plates were stored at 6°C and growth of yeasts was controlled weekly. As a control, an agar plate was prepared with an addition of an appropriate amount of skim milk. Table 7 shows in a summary, that the protective cultures consisting of *Propionibacterium jensenii* SM11 and a *Lactobacillus paracasei* subsp. *paracasei* strain (SM20, SM29 or SM63) still showed unchanged antifungal activities after a storage of 15 weeks at - 80°C.

Table 7

Antifungal activities of selected protective cultures after a storage of 15 weeks at -80°C, incorporated in agar plates.				
Indicator organisms <sup>a</sup>	Protective cultures <sup>b</sup>			
	SM20/SM11	SM29/SM11	SM63/SM11	control
<i>Candida magnoliae</i> 1-35/1	+/-	+/-	+/-	++
<i>Candida parapsilosis</i> 4-5/1 <i>Zygosaccharomyces bailii</i> 1-48/1	-	-	-	(+/-) -
<i>Candida silvicola</i> 4-42/1	-	-	-	++
<i>Candida valida</i> 1-48/8	+	+	+	+
<i>Candida pulcherrima</i> 1-50/13	+/-	+/-	+	++
<i>Candida reukauffii</i> 4-73/4	-	-	-	++
<i>Candida</i> sp. 1-50/15	+	+	+	++
<i>Sporobolomyces salmonicolor</i> 3-46/2	-	-	-	+/-
<i>Candida krusei</i> 3-69/2	-	-	-	-

SM20: *Lactobacillus paracasei* subsp. *paracasei* SM20 (10<sup>8</sup> cfu/ml)

SM29: *Lactobacillus paracasei* subsp. *paracasei* SM29 (10<sup>8</sup> cfu/ml)

SM63: *Lactobacillus paracasei* subsp. *paracasei* SM63 (10<sup>8</sup> cfu/ml)

SM11: *Propionibacterium jensenii* SM 11 (10<sup>8</sup>cfu/ml)

n.d.: not determined

<sup>a</sup> spot-inoculation with 10<sup>6</sup> cfu/ml

<sup>b</sup> incorporated in agar plates

++ strong colony (= 0% inhibition)

+ normal colony (= 25% inhibition)

+/- weak colony (= 50% inhibition)

(+/-) very weak colony (= 75% inhibition)

- no colony (= 100% inhibition)

#### Antifungal activities of protective cultures in a food model

[0122] For a preliminary trial, a food model was set up with different protective cultures. The antagonistic strains were added to tyndallized skim milk at initial levels of 10<sup>8</sup> cfu/g. They were *Lactobacillus paracasei* subsp. *paracasei* SM20, *Lactobacillus paracasei* subsp. *paracasei* SM29, *Lactobacillus paracasei* subsp. *paracasei* SM63, *Lactobacillus plantarum* SM17 and *Lactobacillus plantarum* SM39 in combination with *Propionibacterium jensenii* SM11. Additionally the samples were inoculated with *Candida pulcherrima* 1-50/13 at levels of 10<sup>2</sup> cfu/g and then stored at 6°C. As indicated in Figure 3, the levels of yeasts increased constantly to 10<sup>7</sup> - 10<sup>8</sup> cfu/g in the samples with the *Lactobacillus plantarum* strains SM17 and SM39 as well as in the control sample showing the highest values. Samples containing *Lactobacillus paracasei* subsp. *paracasei* (SM20, SM29 or SM63) showed significant differences. In these samples, *Candida pulcherrima* 1-50/13 increased up to a level of 10<sup>4</sup> cfu/g and then remained almost stable for seven weeks. After four weeks samples with *Lactobacillus paracasei* subsp. *paracasei* SM63 showed an increase in yeasts. The level of propionibacteria and lactobacilli neither increased nor decreased during storage. The pH of samples with protective cultures did not show any significant differences to the control sample (data not shown).

#### Antifungal activities in yoghurt

[0123] A further trial was set up with yoghurt and protective cultures consisting of *Lactobacillus paracasei* subsp. *paracasei* strains SM20, SM29 or SM63 in combination with *Propionibacterium jensenii* SM11. Therefore, a milk enriched with nutrients was used that should favour the growth of contaminating yeasts. This milk was fermented with a commercial yoghurt culture in the presence of a protective culture. The protective cultures were used at concentrations of 10<sup>7</sup> cfu/g or 10<sup>8</sup> cfu/g, respectively. After fermentation and cooling down, the samples were inoculated with a mix of *Candida pulcherrima* 1-50/13, *Candida magnoliae* 1-35/1, *Candida parapsilosis* 4-5/1 and *Zygosaccharomyces bailii* 1-48/1 at a level of 10<sup>2</sup> cfu/g and then stored at 6°C. Figure 4 shows that the levels of yeasts in samples with protective cultures at a concentration of 10<sup>7</sup> cfu/g as well as in the control sample increased constantly. Nevertheless, the control sample always showed the highest values of contaminating yeasts. In all samples containing the protective cultures at a level of 10<sup>8</sup> cfu/g no increase of yeasts was observed. The level of yeasts remained stable over a period of 4 weeks. The same phenomenon was observed with concentrations of 1.7 x 10<sup>8</sup> cfu/g lactobacilli and 5.5 x 10<sup>7</sup> cfu/g propionibacteria (Table 11). In all samples, the levels of protective culture neither increased nor decreased during storage. The pH of samples with protective cultures did not show any significant differences to the control sample (data

not shown). As shown in Table 8, in samples with protective cultures 60 - 70 mg/100 g acetate were enzymatically determined whereas only 4.43 mg/100 g were detected in the control sample without protective culture.

Table 8:

Enzymatically determined concentrations of organic acids metabolised in yoghurt with protective cultures.				
Protective cultures	Organic acids			
	Acetate (mg/100g)	D-Lactate (g/100g)	L-Lactate (g/100g)	Total lactate (g/100g)
SM20/SM11 <sup>a</sup>	68.84	0.01	0.61	0.62
SM29/SM11 <sup>b</sup>	60.95	0.01	0.62	0.63
SM63/SM11 <sup>c</sup>	61.44	0.01	0.65	0.66
control <sup>d</sup>	4.43	0.01	0.74	0.75

SM20: *Lactobacillus paracasei* subsp. *paracasei* SM20

SM29: *Lactobacillus paracasei* subsp. *paracasei* SM29

SM63: *Lactobacillus paracasei* subsp. *paracasei* SM63

SM11: *Propionibacterium jensenii* SM11

<sup>a</sup> SM20,  $1.7 \times 10^8$  cfu/g; SM11,  $5.5 \times 10^7$  cfu/g

<sup>b</sup> SM29,  $1.7 \times 10^8$  cfu/g; SM11,  $2.8 \times 10^8$  cfu/g

<sup>c</sup> SM63,  $1.6 \times 10^8$  cfu/g; SM11,  $8.1 \times 10^7$  cfu/g

<sup>d</sup> no protective culture

[0124] In contrast, the protective culture did not influence the content of D-lactate, L-lactate as well as total lactate (Table 8).

[0125] Using different sensory test, the yoghurts with protective cultures and the control samples without an additional culture did not show any perceptible differences after storage periods of 5 and 15 days.

#### Scale up of the application of a protective culture to yoghurt

[0126] The scale up trials with yoghurt gave similar results as were obtained by the trials on laboratory scale.

#### Antifungal activities on the cheese surface

[0127] Table 9 shows the inhibition of a mix of yeasts on cheese surfaces, treated with three different protective cultures at different concentrations. The cheeses were all contaminated with yeasts to reach a final level on the surface of  $10^2$  cfu/g. The surface of the cheeses was defined as a layer of about 3 mm from the outside of the cube.

Table 9:

Inhibition of yeasts on cheese surfaces held at 6°C.				
	Time (days)	cfu/g of yeasts <sup>a</sup>		
		A	B	C
K1	1	$5.0 \times 10^2$	$3.2 \times 10^2$	$4.1 \times 10^2$
	21	$1.5 \times 10^2$	$< 10^2$	$< 10^2$
K2	1	$5.5 \times 10^2$	$1.0 \times 10^2$	$4.1 \times 10^2$
	21	$3.5 \times 10^2$	$< 10^2$	$< 10^2$
K3	1	$2.2 \times 10^3$	$2.7 \times 10^2$	$1.3 \times 10^3$
	21	$1.3 \times 10^4$	$< 10^2$	$1.0 \times 10^2$
K4	1	$6.3 \times 10^2$		
	21	$8.0 \times 10^3$		

A: inoculated with yeasts and protective culture SM20/SM11

B: inoculated with yeasts and protective culture SM29/SM11

C: inoculated with yeasts and protective culture SM63/SM11

K1: concentration of protective culture:  $10^7$  -  $10^8$  cfu/g surface

K2: concentration of protective culture:  $10^6$  -  $10^7$  cfu/g surface

K3: concentration of protective culture: approx.  $10^6$  cfu/g surface

K4: control, inoculated with yeasts, no protective culture added

a a mix of *Candida pulcherrima* Zygosaccharomyces bailii 1-48/1 1-50/13, *Candida magnoliae* 1-35/1, *Candida parapsilosis* 4-5/1 and

[0128] At concentrations of  $10^8$  and  $10^7$  cfu/g surface, the protective cultures were able to inhibit the outgrowth of yeasts totally during a storage of 21 days at 6°C. The number of yeasts even declined (K1, K2). A level of  $3.0 \times 10^6$  cfu/g surface of lactobacilli and  $3.0 \times 10^6$  cfu/g surface of propionibacteria seemed to be the limit to reach a minimal inhibition of the yeasts. Cheese sample K3A showed lower levels of protective culture and did thus not inhibit the outgrowth of the contaminating yeasts. The levels of protective cultures in the surfaces of the cheeses were in the same range as the cell numbers of the solutions in which the cheeses had previously been soaked (data not shown).

#### Inhibition of *Listeria* in food models with protective cultures

[0129] Table 10 summarises the inhibition of *Listeria* with protective cultures in minced meat and cream.

Table 10:

Inhibition of <i>Listeria</i> by protective cultures in food models held at 6°C and 25°C.					
Food systems	Time (days)	cfu/g of <i>Listeria innocua</i> L17			
		A	B	C	D
Minced meat (6°C)	1	$3.3 \times 10^3$	$2.8 \times 10^3$	$2.6 \times 10^3$	$2.7 \times 10^3$
	28	$6.0 \times 10^2$	$3.7 \times 10^2$	$2.7 \times 10^2$	$4.5 \times 10^2$
	42	$1.7 \times 10^3$	$< 10^2$	$1.5 \times 10^2$	$< 10^2$
	56	$5.1 \times 10^3$	$8.2 \times 10^2$	$< 10^2$	$1.5 \times 10^2$
Minced meat (25°C)	0	$4.1 \times 10^3$	$4.9 \times 10^3$	$4.0 \times 10^3$	$3.7 \times 10^3$
	2	$4.5 \times 10^4$	$5.5 \times 10^3$	$3.0 \times 10^3$	$7.5 \times 10^3$
	5	$6.8 \times 10^4$	$5.9 \times 10^3$	$3.2 \times 10^3$	$5.5 \times 10^3$
Cream (6°C)	1	$2.7 \times 10^3$	$3.2 \times 10^3$	n.d.	n.d.
	14	$1.3 \times 10^3$	$1.2 \times 10^3$	n.d.	n.d.
	21	$1.1 \times 10^3$	$1.5 \times 10^2$	n.d.	n.d.
	28	$1.0 \times 10^3$	$< 10^2$	n.d.	n.d.
Cream (25°C)	1	$2.9 \times 10^2$	$2.6 \times 10^2$	$2.2 \times 10^2$	$3.0 \times 10^2$
	21	$10^7 - 10^8$	$< 10^2$	$< 10^2$	$< 10^2$
		cfu/g of <i>Listeria monocytogenes</i> M1			
		A	B	C	D
Cream (6°C)	1	$1.6 \times 10^3$	$1.7 \times 10^3$	n.d.	n.d.
	7	$2.9 \times 10^7$	$1.9 \times 10^3$	n.d.	n.d.
	14	$3.0 \times 10^8$	$7.5 \times 10^2$	n.d.	n.d.
A: control, inoculated with <i>Listeria</i> no protective culture added B: inoculated with <i>Listeria</i> and protective culture SM20/SM11 C: inoculated with <i>Listeria</i> and protective culture SM29/SM11 D: inoculated with <i>Listeria</i> and protective culture SM63/SM11 n.d.: not determined					

[0130] The two food models were selected to represent high moisture-food with different fat and protein contents and an optimal pH for *Listeria*. Although *Listeria innocua* L17 showed good growth after 21 days on BHI agar stored at 6°C (Table 6), it was not detected in the food models. The viable numbers of the indicator organism, however, decreased in the presence of a protective culture. Furthermore, these samples still showed a nice red meat colour after a storage of 56 days and the control sample was green to brown and clearly spoiled. The pH of the control sample was 6.20 after 56 days that one of the samples with protective cultures in a range of 5.50 to 5.81. The level of propionibacteria and lactobacilli remained unchanged during the storage at 6°C for a period of 56 days. A second charge of minced meat with and without protective cultures was stored at 25°C. After a storage of 5 days, *Listeria innocua* L17 was held in check in the samples with protective cultures ( $3.2$  to  $5.9 \times 10^3$  cfu/g) whereas the viable number of the indicator organism increased to  $6.8 \times 10^4$  cfu/g in the control sample. Additionally, the control sample had totally

gone bad. The cream stored at 6°C showed the same phenomenon after 28 days. In the control sample, the number of *Listeria innocua* L17 was still in the same range as in the beginning ( $10^3$  cfu/g) but in the sample with protective culture, the viable number of the indicator organism went down to under  $10^2$  cfu/g. The level of lactobacilli and propionibacteria neither increased nor decreased during storage. The pH values in the samples with or without protective culture were in the same range. After a storage of 28 days at 6°C the pH of the cream has gone down to 4.78 in the sample without protective culture and to 4.60 in the sample with protective culture. In cream samples stored at 25°C *Listeria innocua* L17 decreased below  $10^2$  cfu/g in the presence of protective cultures but went up to a cell number of  $10^7 - 10^8$  cfu/g in the control sample. Similarly the pH of these samples changed clearly. The samples with protective cultures were in a range of 3.56- 3.68, whereas the control sample still had a pH of 5.80. After 21 days of storage at 25°C, the cell numbers of lactobacilli and propionibacteria were still in the same range as at the beginning. The levels of protective cultures neither increased nor decreased. In the samples with *Listeria monocytogenes* M1 as indicator organism a clear difference in the viable number of the pathogen was observed in the presence or absence of the protective culture. After a storage of 14 days at 6°C, the sample without protective culture contained  $3.0 \times 10^8$  cfu/g of *Listeria monocytogenes* M1 whereas in the sample with the protective culture the number of the indicator organism had even decreased to  $7.5 \times 10^2$  cfu/g (Table 10).

#### Necessary concentration of protective culture in food for a sufficient antimicrobial activity

[0131] To reach a sufficient antimicrobial activity, a certain level of lactobacilli and propionibacteria was necessary. Tables 11 and 12 show the different trials of this study and the corresponding levels of protective culture.

Table 11: Levels of protective cultures used in the different trials to detect antifungal activities (cfu/g).

Cultures	Trials					
	Agarplates <sup>a</sup>		Food Model		Yoghurt <sup>b</sup>	
	LB	PAB	LB	PAB	LB	PAB
SM20/SM11	10 <sup>8</sup>	10 <sup>8</sup>	1.2 x 10 <sup>8</sup>	4.9 x 10 <sup>8</sup>	1.7 x 10 <sup>8</sup>	5.5 x 10 <sup>7</sup>
SM29/SM11	10 <sup>8</sup>	10 <sup>8</sup>	1.1 x 10 <sup>8</sup>	3.2 x 10 <sup>8</sup>	1.7 x 10 <sup>8</sup>	2.8 x 10 <sup>8</sup>
SM63/SM11	10 <sup>8</sup>	10 <sup>8</sup>	9.1 x 10 <sup>7</sup>	4.1 x 10 <sup>8</sup>	1.6 x 10 <sup>8</sup>	8.1 x 10 <sup>7</sup>
					4.3 x 10 <sup>7</sup>	1.5 x 10 <sup>7</sup>
					3.8 x 10 <sup>7</sup>	2.0 x 10 <sup>7</sup>
					1.7 x 10 <sup>7</sup>	1.6 x 10 <sup>7</sup>

SM20: *Lactobacillus paracasei* subsp. *paracasei* SM20

SM29: *Lactobacillus paracasei* subsp. *paracasei* SM29

SM63: *Lactobacillus paracasei* subsp. *paracasei* SM63

SM11: *Propionibacterium jensenii* SM11

LB: lactobacilli

PAB: propionibacteria

n.d. not determined

<sup>a</sup> level of the protective culture was only determined empirically

<sup>b</sup> (1 % of each strain in a 100-fold concentration)

<sup>c</sup> (0.1 % of each strain in a 100-fold concentration)

Table 12: Levels of protective cultures used in the different trials to detect antibacterial activities (cfu/g).

Cultures	Trials									
	Agarplates <sup>a</sup>		Minced meat <sup>b</sup>		Minced meat <sup>c</sup>		Crepe <sup>d</sup>		Crepe <sup>e</sup>	
	LB	PAB	LB	PAB	LB	PAB	LB	PAB	LB	PAB
SM20/SM11	10 <sup>8</sup>	10 <sup>8</sup>	1.1 x 10 <sup>9</sup>	6.3 x 10 <sup>9</sup>	4.8 x 10 <sup>8</sup>	2.9 x 10 <sup>8</sup>	9.3 x 10 <sup>8</sup>	3.8 x 10 <sup>8</sup>	1.0 x 10 <sup>9</sup>	5.7 x 10 <sup>9</sup>
SM29/SM11	10 <sup>8</sup>	10 <sup>8</sup>	1.2 x 10 <sup>9</sup>	8.1 x 10 <sup>9</sup>	4.9 x 10 <sup>8</sup>	4.0 x 10 <sup>8</sup>	n.d.	n.d.	n.d.	n.d.
SM63/SM11	10 <sup>8</sup>	10 <sup>8</sup>	1.6 x 10 <sup>8</sup>	8.7 x 10 <sup>9</sup>	1.2 x 10 <sup>8</sup>	3.0 x 10 <sup>8</sup>	n.d.	n.d.	n.d.	n.d.

SM20: *Lactobacillus paracasei* subsp. *paracasei* SM20

SM29: *Lactobacillus paracasei* subsp. *paracasei* SM29

SM63: *Lactobacillus paracasei* subsp. *paracasei* SM63

SM11: *Propionibacterium jensenii* SM11

LB: lactobacilli

PAB: propionibacteria

n.d. not determined

<sup>a</sup> level of the culture was only determined empirically

<sup>b</sup> minced meat stored at 6°C with *Listeria innocua* L17

<sup>c</sup> minced meat stored at 25°C with *Listeria innocua* L17

<sup>d</sup> crepe stored at 6°C with *Listeria innocua* L17

<sup>e</sup> crepe stored at 6°C with *Listeria monocytogenes* M1

[0132] To reach thus an optimal antifungal activity of the protective culture a level of at least  $1.7 \times 10^8$  cfu/g or cfu/

ml of lactobacilli in conjunction with  $5.5 \times 10^7$  cfu/g or cfu/ml of propionibacteria were necessary (Table 11). On the cheese surface, a level of  $1.0 \times 10^7$  cfu/g surface of lactobacilli and  $5.0 \times 10^6$  cfu/g surface of propionibacteria seemed to be the minimal concentration to reach a constant satisfactory antifungal activity (data not shown). For a sufficient antibacterial activity, the lowest level tested was  $1.2 \times 10^8$  cfu/g or cfu/ml of lactobacilli in conjunction with  $3.0 \times 10^8$  cfu/g or cfu/ml of propionibacteria showing still good inhibitory properties (Table 12). According to hitherto investigations the antimicrobial activities are in the same range as the antifungal.

## References

- [0133] Al-Zoreky, M. H., J. W. Ayres and W. E. Sandine. 1991. Antimicrobial activity of Microgard™ against food spoilage and pathogenic microorganisms. J. Dairy Sci. 74: 758-763.
- [0134] Amann, R. I., W. Ludwig, K.-H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol. Rev. 59: 143-169.
- [0135] Collins, M. D., B. A. Phillips and P. Zanolì. 1989. Deoxyribonucleic acid homolgy studies of *Lactobacillus casei*, *Lactobacillus paracasei* sp. nov., subsp. *paracasei* and subsp. *tolerans* and *Lactobacillus rhamnosus* sp. nov., comb. nov. Int. J. Syst. Bacteriol. 39: 105-108.
- [0136] Daeschel, M. A. 1989. Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technol. 43: 164-167.
- [0137] Daeschel, M. A. 1992. Procedures to detect antimicrobial activities of microorganisms. In Food biopreservatives of microbiological origin. (R. Bibek and M. A. Daeschel eds.). CRC Press, Inc. Boca Raton, Ann Arbor, London and Tokyo. 57-80.
- [0138] Dasen, G., J. Smutny, M. Teuber and L. Meile. 1998. Classification and identification of Propionibacteria based on ribosomal RNA genes and PCR. System. Appl. Microbiol. 21: 251-259.
- [0139] EU-Richtlinie Nr. 95/2/EG des Europäischen Parlaments und des Rats vom 20. Februar 1995 über andere Lebensmittelzusatzstoffe, Farbstoffe und Süßungsmittel.
- [0140] Federal Register. 1988. Nisin preparation: affirmation of GRAS status as a direct human food ingredient. 21 CFR Part 184, volume 53: 11247-11251.
- [0141] Goldenberger, D. 1997. Detection of bacterial pathogens in clinical specimen by broad-range PCR amplification and direct sequencing of part of the 16S rRNA gene. PhD thesis No. 12219, ETH, Zurich, Switzerland.
- [0142] Greisen, K., M. Loeffelholz, A. Purohit and D. Leon. 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J. Clin. Microbiol. 32: 335-351.
- [0143] Grinstead, D. A. and S. F. Barefoot. 1992. Jensenin G, a heat-stable bacteriocin produced by *Propionibacterium jensenii* P126. Appl. Environ. Microbiol. 58: 215-220.
- [0144] Holzapfel, W. H., R. Gelsen and U. Schillinger. 1995. Review Paper. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade-enzymes. Int. J. Food Microbiol. 24: 343-362.
- [0145] Jung, G. 1991. Lantibiotics: a survey. In Nisin and novel lantibiotics (Jung, G. and H.-G. Sahl, eds.). Escom Publishers, Leiden, The Netherlands. 1-34.
- [0146] Lane, D. J. 1991. 16S/23S rRNA sequencing. In: Nucleic acid techniques in bacterial systematics. (Stackebrandt, E. and M. Goodfellow eds.). John Wiley & Sons, New York, USA. 115-176.
- [0147] Miescher, S. 1999. Antimicrobial and autolytic systems of dairy propionibacteria. PhD thesis No. 13486. ETH, Zurich, Switzerland.
- [0148] Pearson, P. W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA 85: 2444-2448.
- [0149] Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74: 5463-5467.
- [0150] Soumalainen, T. H. and A. M. Märyä-Mäkinen. 1999. Propionic acid bacteria as protective cultures in fermented milks and breads. Lait 79: 165-174.
- [0151] Vogel, R. F. 1996. Biotechnology of starter organisms for non-dairy lactic food fermentations (Mini-Review). Adv. Food Sci. 18: 46-51.
- [0152] Ward, L. J. H. and M. J. Timmins. 1999. Differentiation of *Lactobacillus casei*, *Lactobacillus paracasei* and *Lactobacillus rhamnosus* by polymerase chain reaction. Lett. Appl. Microbiol. 29: 90-92.
- [0153] Young, J. P. W., H. L. Downer and B. D. Eardly. 1991. Phylogeny of the phototrophic rhizobium strain BTAi1 by polymerase chain reaction-based sequencing of a 16S rRNA gene segment. J. Bacteriol. 173: 2271-2277.
- [0154] Zusatzstoffverordnung über die in Lebensmitteln zulässigen Zusatzstoffe, vom 26. Juni 1995, Stand am 22. Februar 2000. Eidgenössisches Departement des Innern: Dreifuss. EDMZ, Bern.



**Deposited strains**

**[0155]** The following strains have been deposited on 20.09.2001 with the DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ).

5

- Strain *Propionibacterium jensenii* SM11 has been deposited under the number DSM 14513

- Strain *Lactobacillus paracasei* subsp. *paracasei* SM20 has been deposited under the number DSM 14514.

10

- Strain *Lactobacillus paracasei* subsp. *paracasei* SM29 has been deposited under the number DSM 14515.

- Strain *Lactobacillus paracasei* subsp. *paracasei* SM63 has been deposited under the number DSM 15416.

15

20

25

30

35

40

45

50

55

SEQUENCE LISTING

5 <110> Eidgenössische Technische Hochschule Zürich

<120> Novel biological system with antimicrobial activities  
for the use as natural preservation system

10 <130> Mixture of bacteria

<140>  
<141>

15 <160> 11

<170> PatentIn Ver. 2.1

20 <210> 1  
<211> 25  
<212> DNA  
<213> Artificial Sequence

25 <220>  
<223> Description of Artificial Sequence: PCR-primer

30 <400> 1  
tggctcagaa cgaacgctag gcccg 25

<210> 2  
<211> 20  
35 <212> DNA  
<213> Artificial Sequence

<220>  
40 <223> Description of Artificial Sequence: PCR-primer

<400> 2  
tgcactgaga ttcgacttaa 20

45 <210> 3  
<211> 18  
<212> DNA  
<213> Artificial Sequence

50 <220>  
<223> Description of Artificial Sequence: PCR-primer

55 <400> 3  
caccgagatt caacatgg 18

**EP 1 308 506 A1**

	<210> 4	
	<211> 20	
	<212> DNA	
5	<213> Artificial Sequence	
	<220>	
	<223> Description of Artificial Sequence: PCR-primer	
10	<400> 4	
	tgcatcttga tttaattttg	20
15	<210> 5	
	<211> 19	
	<212> DNA	
	<213> Artificial Sequence	
20	<220>	
	<223> Description of Artificial Sequence: PCR-primer	
25	<400> 5	
	aggaggtgat ccarccgca	19
	<210> 6	
	<211> 18	
30	<212> DNA	
	<213> Artificial Sequence	
	<220>	
35	<223> Description of Artificial Sequence: PCR-primer	
	<400> 6	
	agtttgatcm tggetcag	18
40	<210> 7	
	<211> 18	
	<212> DNA	
45	<213> Artificial Sequence	
	<220>	
	<223> Description of Artificial Sequence: PCR-primer	
50	<400> 7	
	actcctacgg gaggcagc	18
	<210> 8	
55	<211> 17	
	<212> DNA	

<213> Artificial Sequence

<220>

5 <223> Description of Artificial Sequence: PCR-primer

<400> 8

10 accgcggctg ctggcac 17

<210> 9

<211> 22

<212> DNA

15 <213> Artificial Sequence

<220>

20 <223> Description of Artificial Sequence: PCR-primer

<400> 9

ggmttagata ccctggtagt cc 22

<210> 10

<211> 20

<212> DNA

<213> Artificial Sequence

30 <220>

<223> Description of Artificial Sequence: PCR-primer

35 <400> 10

cgttaagtcc cgcaacgagc 20

<210> 11

40 <211> 17

<212> DNA

<213> Artificial Sequence

45 <220>

<223> Description of Artificial Sequence: PCR-primer

<400> 11

50 gtacacaccg cccgtca 17

# Claims

- 55 1. A mixture of bacteria, **characterised in that** said mixture of bacteria is a non starter culture which is free from metabolites and comprises at least one first bacterium selected from the species *Propionibacterium jensenii* and at least one second bacterium selected from the genus *Lactobacillus*.

2. The mixture of bacteria which is a non starter culture according to claim 1, obtainable by the method comprising the steps of:

- Incorporation of a mixture of bacteria which comprises at least one first bacterium selected from the species *Propionibacterium jensenii* and at least one second bacterium selected from the genus *Lactobacillus* and which is free from metabolites into a medium or onto the surface of a medium contained in a first container such that a concentration of at least  $1 \times 10^8$  cfu/ml or  $1 \times 10^8$  cfu/g or  $1 \times 10^7$  cfu/cm<sup>2</sup> of each of said first and second bacteria results;
- Incorporation of contaminants into the medium and/or application onto the surface of the medium of said first container;
- Incorporation into the medium and/or application onto the surface of the medium contained in a second container of the same contaminants as incorporated in the medium and/or applied onto the surface of the medium of said first container, said medium being identical with the medium of the first container but lacking said mixture of bacteria;
- Determination of the inhibition of said contaminants by said mixture of bacteria by storing said first and second container at a suitable temperature during a suitable storage time and by comparing the number and/or concentration of contaminants in the medium and/or on the surface of the medium contained in said first and second container, wherein said inhibiting mixture is such that the number and/or concentration of said contaminants in the medium and/or on the surface of the medium contained in said second container is higher than the number and/or concentration in the medium and/or on the surface of the medium contained in said first container during at least part of said storage time.

3. The mixture of bacteria according to claim 2, **characterised in that** said medium is food or feeding stuff or preferably agar medium.

4. The mixture of bacteria according to claim 2 or 3, **characterised in that** said suitable temperature is approx. 6-25°C, preferably said suitable temperature is at least one selected from the group of approx. 6°C, approx. 12°C, approx. 25°C and said suitable storage time is 7-28 days, preferably said suitable storage time is at least one selected from the group of 7 days, 14 days, 21 days, 28 days.

5. The mixture of bacteria according to anyone of the claims 2-4, **characterised in that** said mixture of bacteria is considered to be inhibiting if the number of the contaminants in the medium and/or onto the surface of the medium contained in said second container is at least log 1, preferably at least log 2, much preferably at least log 3, more preferably at least log 4, even more preferably at least log 5, most preferably at least log 6 higher than the number of the contaminants onto the surface of the medium contained in said first container during at least 3 days, preferably at least 7 days, much preferably at least 14 days, more preferably at least 21 days, most preferably 25 days.

6. The mixture of bacteria according to anyone of the claims 2-5, **characterised in that** said contaminants are fungi or bacteria or mixtures thereof.

7. The mixture of bacteria according to anyone of the preceding claims, **characterised in that** the ratio between said first bacterium and said second bacterium amounts between 1:100 - 100:1, preferably 1:10 - 10:1.

8. The mixture of bacteria according to anyone of the preceding claims, **characterised in that** said second bacterium is selected from the group consisting of *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus plantarum*, and mixtures thereof.

9. The mixture of bacteria according to claim 8, **characterised in that** said second bacterium is selected from one or more strains of *Lactobacillus paracasei* subsp. *paracasei*.

10. The mixture of bacteria according to anyone of the preceding claims, **characterised in that** said second bacterium is selected from the group consisting of *Lactobacillus paracasei* subsp. *paracasei* SM20, *Lactobacillus paracasei* subsp. *paracasei* SM29, *Lactobacillus paracasei* subsp. *paracasei* SM63, and mixtures thereof.

11. The mixture of bacteria according to anyone of the preceding claims, **characterised in that** said first bacterium is *Propionibacterium jensenii* SM11.

12. Food, feeding stuff or medicament **characterised in that** said food, feeding stuff or medicament comprises the

mixture of bacteria according to anyone of the preceding claims.

13. Food according to claim 12, **characterised in that** said food is selected from the group of milk products, preferably sour milk products and/or meat products.

14. Method for manufacturing the food, feeding stuff or medicament, in particular the food according to claim 12 or 13, comprising the steps of:

- Addition of the mixture of bacteria according to anyone of the claims 1-11 during the manufacturing of the food, feeding stuff or medicament such that the concentration of said at least one first bacterium and said at least one second bacterium in the food, feeding stuff or medicament amounts each at least to  $1 \times 10^8$  cfu/ml or each at least to  $1 \times 10^8$  cfu/g of the food, feeding stuff or medicament or each at least to  $1 \times 10^7$  cfu/cm<sup>2</sup> of the surface of the food, feeding stuff or medicament;
- Controlling the manufacturing parameters during the manufacturing such that said concentration of said mixture of bacteria remains constant.

15. Method according to claim 14, **characterised in that** the concentration of said first bacterium in the food, feeding stuff or medicament amounts at least to  $1 \times 10^9$  cfu/ml or at least to  $1 \times 10^9$  cfu/g of the food, feeding stuff or medicament or at least to  $1 \times 10^8$  cfu/cm<sup>2</sup> of the surface of the food, feeding stuff or medicament and said second bacterium amounts at least to  $1 \times 10^8$  cfu/ml of said food, feeding stuff or medicament or at least to  $1 \times 10^8$  cfu/g of said food feeding stuff or medicament or at least to  $1 \times 10^7$  cfu/cm<sup>2</sup> of the surface of said food, feeding stuff or medicament, or vice versa.

16. Method according to claim 14 or 15 comprising one or more fermentation steps, **characterised in that** said mixture of bacteria is added before said one or more fermentation steps and/or between said more fermentation steps and/or after said one or more fermentation steps.

17. Method for storing the food, feeding stuff or medicament of claims 12 or 13 or the food, feeding stuff or medicament manufactured according to anyone of the claims 14-16 comprising the step of:

Controlling the storage parameters during the storage such that said concentration of said first and said second bacterium remains constant.

18. Use of the mixture of bacteria according to anyone of the claims 1-11 to inhibit microorganisms selected from the group consisting of fungi, bacteria and mixtures thereof that are pathogenic and/or spoilers, in particular in food, feeding stuff or medicaments.

19. Bacteria selected from the group consisting of *Propionibacterium jensenii* SM11, *Lactobacillus paracasei* subsp. *paracasei* SM20, *Lactobacillus paracasei* subsp. *paracasei* SM29, *Lactobacillus paracasei* subsp. *paracasei* SM63, or mixtures thereof.

20. Food, feeding stuff or medicament, **characterised in that** said food, feeding stuff or medicament comprises at least one bacterium that is a non starter culture that is free from metabolites from said at least one bacterium and wherein said at least one bacterium is selected from the genus *Lactobacillus* or from the species *Propionibacterium jensenii*, preferably said bacteria is a bacteria as described above.

21. Method for manufacturing and/or storing food, feeding stuff or medicament, in particular the food, feeding stuff or medicament according to claim 20, comprising the steps of:

- Addition of the at least one bacterium as defined in claim 20 during the manufacturing of the food, feeding stuff or medicament such that the concentration of said at least one bacterium in the food, feeding stuff or medicament amounts at least to  $1 \times 10^8$  cfu/ml or at least to  $1 \times 10^8$  cfu/g of the food, feeding stuff or medicament or at least to  $1 \times 10^7$  cfu/cm<sup>2</sup> of the surface of the food, feeding stuff or medicament;
- Controlling the manufacturing parameters during the manufacturing such that said concentration of said at least one bacterium remains constant;
- Controlling the storage parameters during the storage such that said concentration of said at least one bacterium remains constant.

Fig. 1

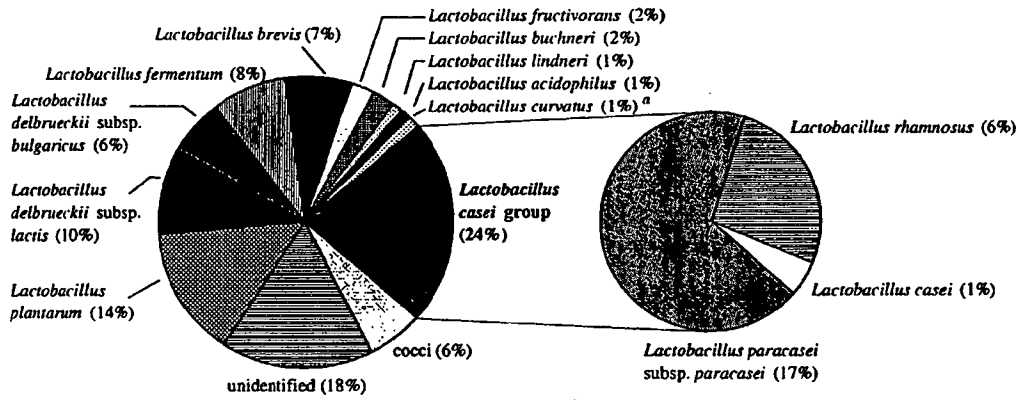


Fig. 2

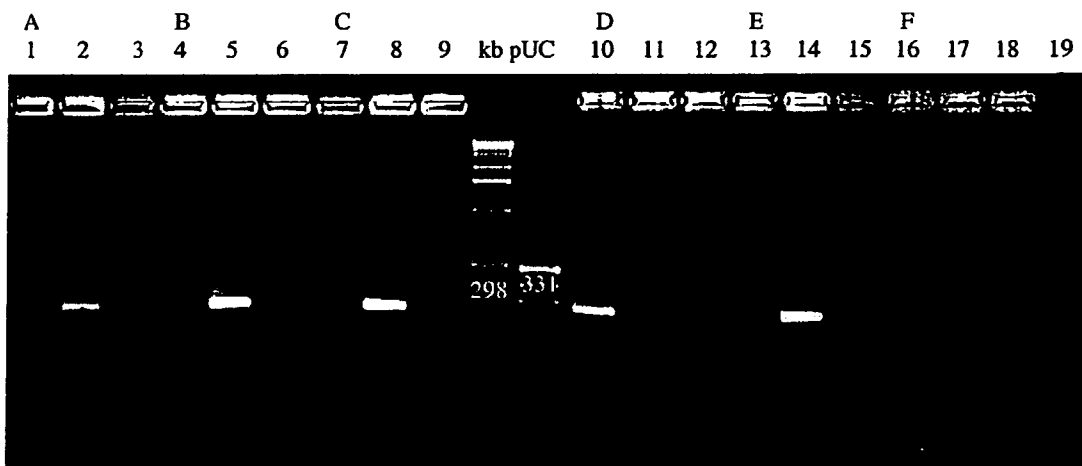


Fig. 3

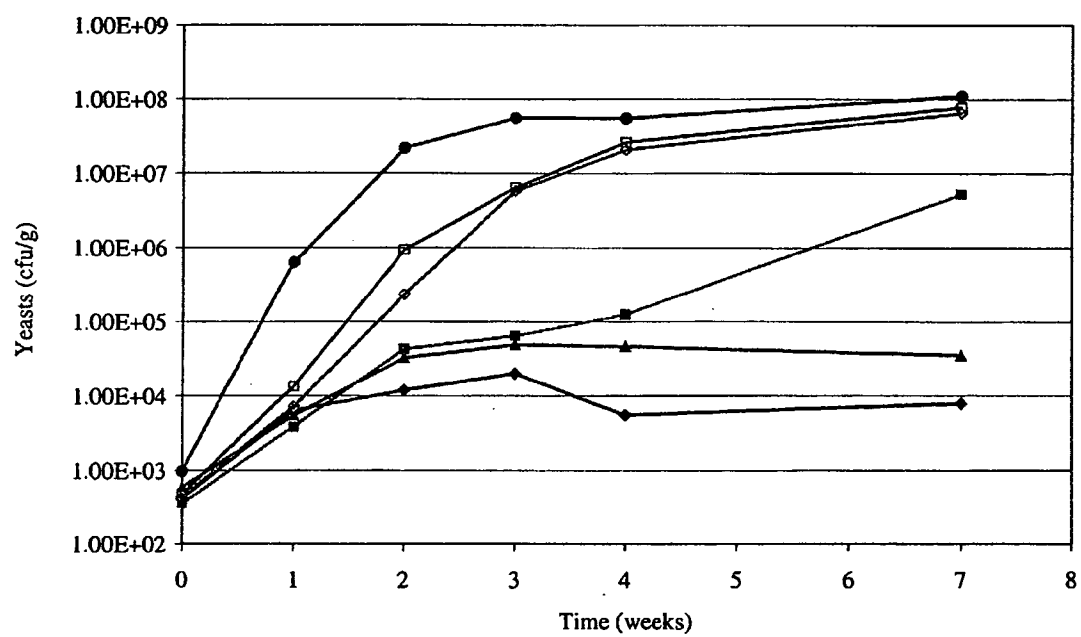
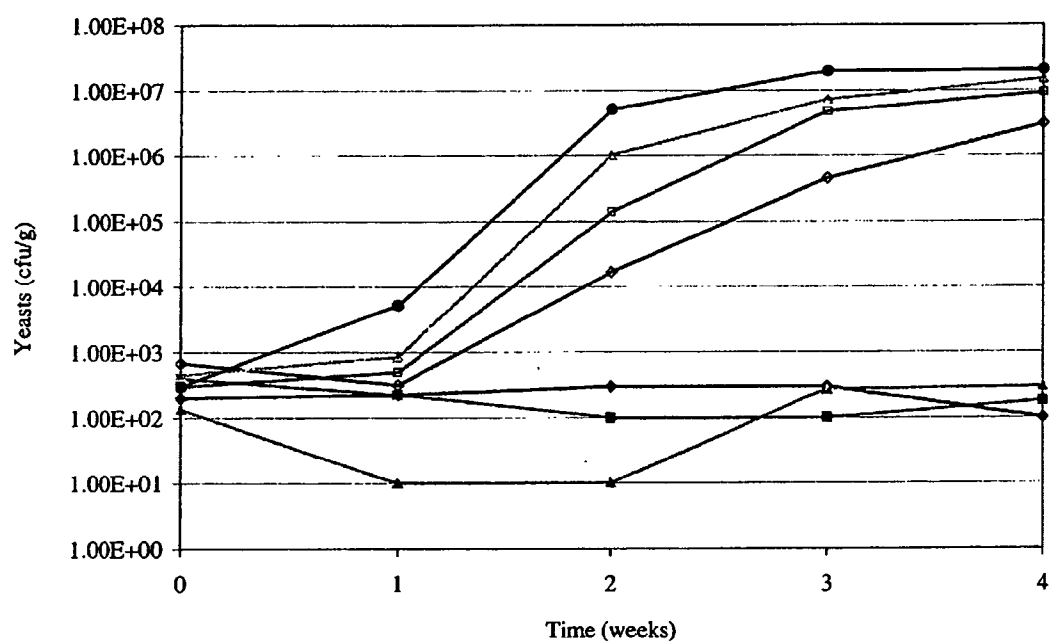




Fig. 4





European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number  
EP 01 12 5464

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
D,X	SUSANNE MIESCHER: "Antimicrobial and Autolytic Systems of dairy Propionibacteria" 1999, SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH (ETHZ), ZURICH, SWITZERLAND XP002192334 p. 58-62, p. 79-83 Diss. ETH No. 13486	1-21	C12N1/20 A01N63/00 A23K3/00 A23L3/3571 C12R1/245 C12R1/01
X	US 4 981 705 A (TOMES NANCY J) 1 January 1991 (1991-01-01)	20,21	
Y	* the whole document *	1-18	
D,Y	EP 0 576 780 A (VALIO OY) 5 January 1994 (1994-01-05) * abstract *	1-18	
Y	P. JAVANAINEN AND Y.-Y. LINKO: "Factors affecting rye sour dough fermentation with mixed-culture pre-ferment of lactic and propionic acid bacteria" JOURNAL OF CEREAL SCIENCE, vol. 18, 1993, pages 171-185, XP002192333 * abstract *	1-18	
			TECHNICAL FIELDS SEARCHED (Int.Cl.7)
			A01N A23L A23K C12R
The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 7 March 2002	Examiner Stolz, B
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document			

EPO FORM 1503 03.82 (P04C01)

**ANNEX TO THE EUROPEAN SEARCH REPORT  
ON EUROPEAN PATENT APPLICATION NO.**

EP 01 12 5464

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

07-03-2002

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
US 4981705	A	01-01-1991	AT 400282 B	27-11-1995
			AT 79590 A	15-04-1995
			BE 1002673 A3	30-04-1991
			CZ 9005193 A3	15-11-1995
			DD 293483 A5	05-09-1991
			DE 4009999 A1	08-05-1991
			DK 68090 A	07-05-1991
			FR 2653973 A1	10-05-1991
			GB 2237723 A, B	15-05-1991
			JP 2516452 B2	24-07-1996
			JP 3147751 A	24-06-1991
			NL 9000676 A	03-06-1991
			NO 177843 B	28-08-1995
			SK 519390 A3	11-01-1999
EP 0576780	A	05-01-1994	FI 922699 A	11-12-1993
			AT 173502 T	15-12-1998
			DE 69322131 D1	24-12-1998
			DE 69322131 T2	20-05-1999
			DK 576780 T3	02-08-1999
			EP 0576780 A2	05-01-1994
			ES 2125278 T3	01-03-1999
			US 5378458 A	03-01-1995

EPO FORM P0459

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82